IDENTIFICATION OF THE FIRST RECEPTOR FOR A PREGNANCY SPECIFIC GLYCOPROTEIN. TETRASPANINS FIND THEIR LIGAND

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ABSTRACT

Identification of the First Receptor for a Pregnancy Specific Glycoprotein. Tetraspanins find their Ligand.

Roseann Marie Waterhouse, Doctor of Philosophy, 2001

Thesis Directed by Gabriela Dveksler, Associate Professor, Department of Pathology

Pregnancy specific glycoproteins (PSGs) are a family of secreted proteins produced by the placenta, which have been shown to be essential for pregnancy success. The ability of human PSGs to induce anti-inflammatory cytokines has led to the hypothesis that these proteins may function to protect the fetus from attack by the maternal immune system. With the purpose of developing an animal model to study the function of PSGs, we have studied the effects of murine PSG17 on macrophages and we have cloned its receptor. RAW 264.7 cells and peritoneal macrophages were treated with recombinant PSG17N, which consists of the N-domain of PSG17. PSG17N induced production of IL-10 and IL-6 at the protein and RNA levels in these cells. Secretion of TGFβ1 and PGE_2 was also induced upon treatment with PSG17N. We then examined the PSG17-RAW cell surface binding interaction. Scatchard analysis revealed that there are approximately 1770 binding sites per cell with a Kd of 2.2×10^{-11} M. For the purpose of cloning the PSG17 receptor, we screened a RAW cell cDNA library by panning. The receptor was identified as CD9, a member of the tetraspanin superfamily. The specificity of the CD9-PSG17 interaction was confirmed by ELISA and flow cytometry in CD9-transfected cells. Furthermore, binding of PSG17 to CD9-expressing cells was blocked with anti-CD9 antibodies. We then tested whether murine PSG18 and 19, which share with PSG17 the ability to induce cytokines in macrophages, use CD9 as their receptor. These proteins do

not bind to CD9. In conclusion, we have identified the first receptor for a PSG as well as the first natural ligand for a member of the tetraspanin superfamily.

IDENTIFICATION OF THE FIRST RECEPTOR FOR A PREGNANCY SPECIFIC GLYCOPROTEIN. TETRASPANINS FIND THEIR LIGAND

By

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Table of Contents

List of Figures

(Figures in Paper #2 continued)

I. Introduction

Significance

Pregnancy specific glycoproteins (PSGs) are a family of secreted proteins produced by the placenta. They were discovered in 1971 in the blood of pregnant women [1]. PSGs are first detected 3 to 4 days post fertilization, coinciding with implantation of the blastocyst into the uterine wall [2]. Maternal serum concentration of these proteins increases exponentially during pregnancy reaching levels of 200-400 μg/ml at term [3, 4], making PSGs the most prevalent "pregnancy–related" protein in maternal serum. Reduced expression of these proteins correlates with fetal hypoxia, fetal growth retardation, pre-eclampsia, and spontaneous abortion [5-8]. Despite their identification thirty years ago, *in vitro* biological functions for PSGs have only been identified in the past few years [9-12], and their physiological role in pregnancy has yet to be defined. The research reported in these manuscripts contributes to the accumulating knowledge regarding PSG immuno-regulatory function *in vitro,* and, importantly, this is the first report to identify a receptor for a PSG.

The PSG Family is a Subfamily in the Carcinoembryonic Antigen Family

Screening of human placental cDNA libraries revealed that the pregnancy specific glycoprotein genes are highly homologous [13-15]. The PSG gene family is classified by sequence homology as a subfamily in the Carcinoembryonic antigen (CEA) gene family. The CEA gene family belongs to Immunoglobulin (Ig) superfamily [16]. Transcripts of CEA gene family members code for proteins containing repeating domains each consisting of β-sheet structure and the conserved disulfide bridges of an Ig fold, all

characteristics of this superfamily. The CEA gene family can be subdivided into the CEA/ nonspecific cross-reacting antigen (NCA) subfamily and the PSG subfamily. The PSG subfamily only contains pregnancy specific glycoproteins, while the CEA subfamily is comprised of carcinoembryonic antigens (CEA), biliary glycoproteins (BGP), nonspecific cross-reacting antigen (NCA) and six other CEA family members (CGMs) (reviewed in [17-19]). All 29 members of the human CEA gene family are localized to chromosome 19 [20, 21]. There are 12 CEA/NCA genes, 11 PSG genes [22] and 6 CGMs (CGM13-18) [23]. Four of the 12 CEA genes are pseudogenes while none of the CGM genes have shown to be expressed. Importantly, there are no known pseudogenes in the human PSG family and expression of all 11 PSG genes has been reported [23, 24].

Gene and Protein Structure for Human CEA family members

The members of the CEA gene family have similar gene organization. The first exon (designated L/N) codes for the 5'UTR and part of the leader signal peptide (21 aa). Exon two encodes the remainder of the signal peptide and the first domain of the mature protein, termed the "N-domain" (107-110 aa). This domain is homologous to Immunoglobulin variable (IgV) domains. Additional domains, which are Ig-constant (IgC) -like, are encoded by single exons. These IgC-like domains are designated "A" (92 aa) and "B" (86 aa). The number of IgC-like domains is gene-specific, ranging from zero to six. Each IgC-like domain contains at least two cysteine residues, which are assumed to stabilize the immunoglobulin-like fold by forming a disulfide bridge. Significant nucleotide and amino acid conservation among the internal repeat domains of PSG and

CEA genes suggests that both families evolved recently by the duplication of a single primordial gene [25].

The CEA/PSG subfamily division also reflects some differences in mature protein structure. The proteins from both subfamilies are structurally similar, containing multiple Ig-like domains and are highly glycosylated, however, CEA/NCA proteins are attached to the plasma membrane by a hydrophobic transmembrane region at the C' terminus, or through a covalent linkage to membrane-bound glycosylphosphatidyl-inositol [26]. However, in PSGs, a truncated, largely hydrophilic region replaces this C-terminal domain, dictating secretion of the proteins by the cell [18]. Slight differences in post-translational modification of CEA family members further indicate separation between the two subfamilies [25]. At least 30% of the molecular weight of PSGs can be attributed to the addition of carbohydrate residues, whereas the total glycosylation of other CEA family members can range from 30-60% [17]. In contrast to the differences between subfamilies, the N-domain of CEA gene family proteins are highly conserved within each subfamily, exhibiting over 90% nucleotide sequence similarity [27]. (N-domain alignments and diagrams of human, mouse, rat and guinea pig CEA and PSG subfamily structural domains can be viewed at the CEA homepage www.ukl.uni-freiburg.de/immz/cea/.) Nucleotide sequence homology between the two CEA subfamilies is less conserved in the N domain (65- 75%). The N-domains of PSG, NCA, and CEA proteins mediate binding to their putative ligands, which implies this domain is critical for biological function [11, 12, 28, 29].

Expression of Human Pregnancy Specific Glycoproteins

1. Sites of expression

Human PSGs are primarily expressed by syncytiotrophoblasts [30] and PSG transcripts have also been identified in amnionic and chorionic tissue [31], which suggests that these proteins play a key role in pregnancy. Although the most abundant source of PSGs is the placenta [32], expression of PSG transcripts is not limited to pregnancy [33]. Expression of human PSG transcripts has also been described in intestine [34], testis [35], uterus [36], salivary gland [37], endometrial tissue [10], bone marrow plasma [38], and breast tumors [39, 40]. The presence of PSGs has been reported in specific cell types including fibroblasts in culture [41, 42], myeloid cell lines [43, 44], on the surface and in cytoplasm of granulocytes [45], and in cells of the myelomonocytic lineage [38]. PSGs are produced by trophoblastic tumors and non-trophoblastic tumors such as choriocarcinoma and breast cancer [39, 46, 47]. PSG transcripts have been cloned from first trimester fetal liver, however PSG mRNA has not been isolated from adult liver [48]. The function of PSGs outside of pregnancy is unknown. Further analysis of PSG function and receptor location may bring to light the significance of these external sites of expression.

With primary sequence conservation between all members of the CEA family, the accuracy of some immunological methods used to identify expression sites is questionable. Experiments using anti-sera including immunoprecipitation, immunohistochemical staining and Western blotting may be flawed as antibodies to CEA family proteins have been shown to cross-react between family and subfamily protein

members [18, 49]. In addition, oligonucleotide probes have been shown to cross-react with CEA and NCA family members [50].

2. Human PSG Structure and function

Each human PSG transcript encodes a 34 amino acid (aa) leader (L) that is cleaved upon secretion of the protein, an N-terminal IgV-like domain (108-109 aa) designated "N", two or three internal IgC-like domains [A1/A2 (93aa) and/or B1/ B2 (85aa)] and a C-terminal domain (C) (2 –81 aa) [20]. PSG genes are alternatively spliced leading to variation in domain structure for individual PSGs. PSG mRNA organization is primarily either L-N-A1-A2-B2-C, L-N-A1-B2-C, or L-N-A2-B2-C [23]. All PSG polypeptides have 324 to 435 amino acid backbones with multiple potential N-linked glycosylation sites. Although splice variants exist, only three distinct serum PSG fractions of 72, 64, 58 kilodaltons have been identified by gel electrophoresis [15]. Human PSGs contain only one Ig-variable-like domain, the N-domain, while the A and B domains are all IgC-like. Excluding the C-terminus, the nucleotide sequences of human PSGs are 90% homologous [20].

As is characteristic of the CEA family members, the PSG N-domain was suspected to be responsible for binding of these proteins to their ligands. The N-terminal region of recombinant PSG6 has been shown to be sufficient for binding to human monocytes [12]. In addition, a 20 amino acid peptide derived from this domain binds to promonocytic cells *in vitro* [51] suggesting that this domain is essential for PSG function. The arginine–glycine-aspartic acid (RGD) sequence motif is found at a conserved position within the N-domain of PSG 2, 3, 5, 6, 7, 9 and 11. The significance of this

motif remains controversial. RGD motifs are present in several proteins with integrin ligands such as fibrinogen, vitronectin, and fibronectin [52]. Based on the presence of this motif, proposed biological activities for PSGs have included cell-mediated binding with extracellular matrix [13, 53], growth factor inhibition [25, 54], and inhibition of cell migration [53]. To date none of these functions has been demonstrated for PSGs *in vivo* or *in vitro*.

However there is evidence *in vitro* to support an immuno-regulatory function for PSGs. In one study, PSG11 which contains an RGD motif, but not PSG1 (which does not contain an RGD sequence), increased IL-10 production by LPS treated primary monocytes and the promonocytic cell lines, U937 and THP-1, leading one research group to suggest that the presence of the RGD motif in PSG11 was essential for binding and biological function [51]. In contrast, our laboratory has demonstrated that both PSG11 and PSG1 have similar biological functions. Both of these proteins as well as PSG6 induced production of IL-6, IL-10 and TGF β_1 by human elutriated monocytes *in vitro*, albeit with different kinetics [12]. The disparity between our results and those of Rutherford can potentially be explained by the quantity of protein used in each study. Our results indicate that higher concentrations of PSG1 and PSG11 were necessary to induce cytokines production in these cells. The high levels of PSG expression during pregnancy, together with their influence on monocyte cytokine production are consistent with the proposed biological function of fetal protection from maternal immune rejection [55, 56].

PSGs have also been suggested to have a role in embryogenesis. Murine embryos co-cultured with recombinant human PSG1 developed rapidly into morulae and

blastocysts [57]. However, incubation with recombinant PSG3 did not increase cellular proliferation. These results suggest that PSG1 and PSG3 play different roles in fetal development, and that some human PSGs may cross-react with murine ligands. Further evidence of human-murine cross-reactivity has been obtained from experiments where recombinant human PSGs induced IL-10, IL-6 and TGFβ_1 in a mouse macrophage cell line (RAW 264.7)[12]. Because differential temporal expression of PSG proteins during human pregnancy cannot be easily determined, a proliferative function for PSG1 early in gestation may or may not be possible in human fetal development.

Outside the context of pregnancy, low baseline PSG expression levels of less than 0.5 μg/L have been measured in the serum of men and non-pregnant women [32, 58]. The presence of PSG transcripts in fetal liver and adult bone marrow is suggestive of a role for these proteins in hematogenesis. The developing liver is the primary site of fetal hematopoiesis during weeks 10-31 of development, while bone marrow is the primary location of adult hematopoiesis. Fetal liver and adult bone marrow PSG expression implies potential involvement of these proteins in hematopoietic differentiation or cell growth. In a study aimed to determine the *in vivo* hematopoietic effects of PSGs, bone marrow was transplanted into irradiated mice and then purified human PSGs were injected at various doses for 20 days [9]. The mice were bled every 2 days and cell counts revealed that the groups treated with 13.9 or 139 nM had an increased platelet count and a quicker white cell recovery rate compared to mice treated with control protein. Unfortunately, a mixture of PSGs was used in these experiments, making it is impossible to determine whether this effect was due to a single PSG protein or a

combination of PSGs. However, the results suggest that placental PSGs enhance platelet and white blood cell counts supporting a role in hematogenesis.

With a variety of expression sites and potential functions, human PSGs may bind to one or more putative receptors, however a specific molecular PSG binding partner has not been identified. A peptide derived from PSG11, containing the RGD motif, has been shown to bind to cells of the myeloid lineage, but not to B or T cell lines [51]. Human monocytes treated with PSG1, PSG6 and PSG11 induced a biological response, the production of Th2 cytokines [12]. Thus monocyte/macrophage cells express one or more receptors for human PSGs. Crosslinking and immunoprecipitation experiments to characterize the putative receptor on promonocytic THP-1 cells revealed a binding product of 46 kilodaltons [51]. Whether this 46 kDa protein is a receptor for human PSGs and is sufficient to trigger signaling mechanisms necessary for cytokine induction or other putative PSG functions remains unknown.

Murine Pregnancy Specific Glycoproteins

1. Sites of expression

Murine PSGs are expressed by primary and secondary trophoblast giant cells of mouse placenta early in gestation and then later by spongiotrophoblast cells and their precursors. All murine PSGs are co-expressed by placental tissue 10-17 days post implantation [59], but expression at earlier times post-fertilization can not be ruled out. RT-PCR analysis of adult murine tissues including kidney, lung, testis, ovary, liver, brain, thymus, heart and spleen did not identify transcripts for murine PSG17-19, 21, 23

and 28 [11, 60, 61]. The concentration of murine PSG proteins in serum from pregnant mice is not known.

2. Structure and function

Screening of a YAC library revealed that there are 14 murine PSG genes. The genes have been identified based on partial genomic sequence of the N domain. These genes are mapped to chromosome 7 by fluorescent in-situ hybridization and are expressed exclusively in the placenta [11, 60, 61]. The only murine PSG pseudogene is Cea6, which correlates with a deletion of one nucleotide in the leader sequence, resulting in a shift in the reading frame [60]. Chromosome 7 is syntenic to human chromosome 19 where human PSGs are located [62]. It is difficult to experimentally distinguish between different murine gene family members as many PSG genes and mRNAs have not been completely sequenced (Dveksler & Zimmerman, *personal communication*). However, full-length cDNAs have been identified for PSG17-19 [60], PSG23 and PSG28 [61]. In these genes, as is characteristic of human PSGs, each exon codes for an individual protein domain. All murine PSGs characterized so far contain four Ig-like domains [60, 63]. Murine PSGs differ from human PSGs in domain structure, containing three Ig variable-like regions and a single Ig-constant-like domain. The amino acid homology of the murine PSG N-domains varies between 54-93%. Similar to the human N-domain RGD motif, a highly conserved glycine residue flanked by one of four amino acids (R/H-**G**-E/K) is found at a conserved site in the murine N-terminal domain [60]. This motif is present in all murine PSGs with the exception of PSG19, in which the tri-peptide asparagine-glycine-lysine (NGK) is found at this site.

Our laboratory was the first to report a biological function for a murine PSG [11]. In an *in vitro* study, murine macrophages were treated with a recombinant GST-PSG18 fusion protein, produced using a baculovirus expression system. Analysis of levels of RNA for the TH2 cytokine Interleukin-10 was performed. Treatment with both PSG18 and a truncated form of the protein consisting of only the N-domain (PSG18N) increased levels of IL-10 transcripts, with a peak at 2 hours post treatment. The increase in RNA expression correlated with an increase in IL-10 protein secretion as measured by ELISA. Dose response curves showed that the level of IL-10 protein steadily increased until it reached a plateau at 222 nM of PSG18N. To confirm that the increase in IL-10 secretion was not the result of priming macrophages with contaminating inflammatory agents such as LPS, induction of IL-1β, TNFα, iNOS, and IL-12 mRNA expression was also examined. PSG18 treatment did not increase expression of these cytokines, indicating that the induction of IL-10 was a specific effect. The results of these experiments suggest that the biological effect of PSG18 is similar to human PSG1, PSG6 and PSG11 [12] and more crucial functions for PSGs may yet be found. Whether induction of macrophage cytokine secretion is a function of other murine PSGs, or whether murine PSGs have individual specialized functions has not yet been studied.

A Murine Model for PSG Function

Although CEA family members have 50-60% sequence similarity, identification of rodent and human orthologues for each member of the PSG subfamily has not been possible. Although this complicates the evaluation of data from a murine model of PSG function, investigation of PSGs *in vivo* and at the fetal level (for obvious ethical reasons)

must be carried out in a well-characterized animal system. Both human and mice are hemochorial placentation mammals; maternal blood does not come into direct contact with fetal blood due to a layer of chorionic tissue surrounding fetal blood vessels. Therefore, murine PSG studies can indicate potential cell interactions and reveal putative ligands for these proteins, which could greatly facilitate understanding of the function of human PSGs. Furthermore, human PSGs have been shown to interact with murine macrophage cell lines producing similar biological effects and suggesting functional conservation between these species [12, 57]. Importantly, the complex steps involved in embryonic implantation and development have been more thoroughly defined in mice (reviewed by [64]), making these animals an ideal choice for study of PSG biological function.

Pregnancy Specific Glycoproteins in Other Animal Species

In addition to humans and mice PSGs have been identified in the rat [65, 66], cow [67], monkeys [32], chimpanzee, orangutan [68], and most recently, baboon [69]. Streydio *et al* (1990) analyzed PSG gene development and concluded that the evolution of the PSG gene family appears to coincide with the divarication of mammals, thereby, coordinating placental development with expression of PSGs [20]. The similarities between CEA proteins and genes in rodents and primates have been reviewed [27]. Of particular note, all expressed PSG/CEA members across species contain an identical number of amino acids in the leader peptide. Outside of this region, interspecies comparison between PSGs has been more difficult. Computer analyses indicate that a parallel but independent evolution of PSGs occurred as these two mammalian orders,

primates and rodentia, diverged perhaps resulting in the differing numbers of IgV and IgC-like domain, and RGD-like motifs observed in mouse and human PSGs today [20, 27].

Placental PSGs: Expression of human PSGs in normal pregnancy and in cancer

During pregnancy, human PSGs are produced by syncytiotrophoblast [39], a multinuclear syncytial cell layer of the placenta, which separates fetal and maternal blood. *In vitro*, human embryo cultures secrete PSGs from three to four days post fertilization [2]. Because of this early expression, PSGs are suspected to be involved in trophoblastic invasion of the uterus [14]. *In vivo*, PSGs are detectable in the maternal serum seven days post conception. Blood samples obtained at two-week intervals over the course of pregnancy revealed that PSG serum concentration continues to rise throughout the forty weeks [70]. It is interesting to note that human pregnancy specific glycoproteins are the only pregnancy related proteins whose concentration continue to rise throughout pregnancy until birth [3]. PSG serum concentration reaches 200-400 μg/ml at term [3]. The half-life of these proteins in maternal blood after parturition is longer than other pregnancy related proteins at a value of 20-40 hours [71]. The high serum concentration and long half-life indicate that the serum concentration of these proteins returns to baseline levels within weeks of childbirth [70].

Evidence supporting the necessity of placental PSGs can be implied from the observation that PSG maternal serum concentration, significantly lower than the median, is predictive of spontaneous abortion [8]. Low maternal serum concentration of PSGs correlates with intrauterine growth retardation, preeclampsia and fetal hypoxia [6, 7, 72].

Furthermore, a chronic reduction in expression of PSG11 transcripts is observed in endometrial tissue of women who suffer from recurrent spontaneous abortion [10]. The importance of PSGs during pregnancy is also manifested in experiments using animal models. Injection of anti-human PSG antibodies into monkeys induced abortion in these animals [73]. Spontaneous abortion also occurred when pregnant mice were injected with rabbit anti-murine PSG antibodies [74].

Excessive serum levels of PSGs are associated with choriocarcinoma, invasive mole, hydatiform mole (PSG6) and ovarian cancer [46, 75-77]. Serum PSG levels are used clinically to follow the progression of gestational trophoblastic diseases. PSG quantitation assists in detection of ectopic pregnancies. In these cases, PSGs are identified in maternal serum earlier than normal, before seven days, but at significantly lower levels [78]. In normal pregnancy, maternal serum contains multiple species of PSGs, however it is not known whether different protein species are co-expressed at similar levels throughout pregnancy, or if there is a pattern coordinating development with expression of different PSG proteins [38]. Two groups, Wu *et al* (1993) and Chamberlain *et al* (1994) reported differential expression of human PSG transcripts by the placenta using RT-PCR and primers specific for the various PSG cDNAs [54, 79]. However, it is difficult, if not impossible, to correlate their mRNA expression findings to protein expression, due to antibody cross-reactivity between PSG proteins.

A Brief Overview of the Multifaceted Phenomenon of Fetal Tolerance during Pregnancy

The fetus expresses both maternal and paternal genes, and therefore exhibits non-maternal cell surface antigens. From this point of view, the comparison of the fetus to a semi-allogeneic graft [80] is indisputable, however, controversy stems from contentions over the mechanisms required by the maternal immune system to maintain or tolerate the "fetal allograft." Medwar and Billingham proposed the following four hypotheses to explain the lack of fetal rejection: the fetus is non-immunogenic, the conceptus represses maternal immune responses, the uterus is an immune-privileged site, and the placenta generates an anatomic immune partition between the mother and the unborn child [81]. Experimental evidence has already refuted the first three hypotheses. Although limited, the fetal placental unit does have immunogenic properties [82-84]. The maternal immune system is not repressed during pregnancy as women can mount an immune response to infection by various pathogens. Introduction of foreign skin grafts into the uterus of laboratory animals resulted in graft versus host symptoms and revealed that the uterus is not an immune privileged site as the grafts were infiltrated by maternal immune cells [85]. Several mechanisms thought to play a role in the regulation of "fetal immune privilege" are reviewed here. All discussed tolerance mechanisms can be ascribed to the fourth hypothesis. The first mechanism is described in more detail, as it is the one that is most directly linked to current known PSG biological functions. Individual immuno-regulatory mechanisms during pregnancy may function alone but it is likely that several mechanisms co-exist, and should thus be viewed as a cluster of overlapping events necessary for fetal survival.

Anti-inflammatory Th2-Type Immune Response During Pregnancy as a Tolerance Mechanism

Both placental and uterine tissues produce and respond to cytokines, and these interactions can dramatically affect pregnancy outcome. Placental and uterine macrophages are a source of transforming growth factor (TGF) β, interleukin (IL)-6 and tumor necrosis factor (TNF) α [86]. Uterine cells can generate TNFα [87], TGFβ [88], and granulocyte colony stimulating factor (G-CSF)[89]. The uterus expresses IL-6 after implantation [90], IL-8 [91], and other cytokines during and after implantation [92]. Trophoblasts produce both anti-inflammatory and inflammatory cytokines including IL-1β [93][[94], TNFα, IL-6 [95-97], IL-8, IL-4, IL-10 [98, 99] and TGFβ [94, 100]. These bioactive molecules can act locally in a paracrine or autocrine manner, or at distal systemic sites regulating growth, development, and function of various tissues [101].

Cytokine production patterns by T helper (TH) cells have been characterized into three categories: TH0, TH1 or TH2. TH0 cells are precursor cells that can differentiate into either TH1 or TH2 in response to signals from antigen presenting cells (APC). In the presence of IL-4, TH0 cells become TH2 cells, which secrete IL-4, IL-5, IL-6, IL-10 and TGF-β. Secretion of IL-12 or IFN-γ during antigen presentation results in production of TH1 cells. These cells produce IL-2, TNFα, IL-12 and interferon (IFN)-γ. IL-10 can down-regulate the production of TH1 cytokine synthesis while the adverse effect, an inhibition of TH2 cytokine synthesis, occurs in the presence of IFN-γ [102]. The products of both of these TH cell types can generate an immune response polarized toward TH1 or TH2, if large quantities of the cytokine are present. In the case of a predominance of TH2 cell products, an anti-inflammatory environment associated with a humoral or antibody-mediated immune response is cultivated. In contrast, abundant TH1

cytokines stimulate a primarily inflammatory immune environment or cell-mediated immune response.

Precise patterns of cytokine expression are critical in regulating immune system cells. A TH1 immune environment has been shown to be detrimental to pregnancy in both mice and humans. IL-2, TNFα and IFNγ play important roles in abortion. Injections of these inflammatory cytokines terminate normal murine pregnancies [103-106]. Spontaneous abortion can be induced with LPS which upregulates production of inflammatory cytokines [107]. Additional evidence associating TH1 immune responses with harm to pregnancy is observed in studies of a mouse model of recurrent spontaneous abortion (RSA). Abortion occurs more frequently in CBA/JXDBA/2 matings (50%) in comparison to normal (CBA/J X BALB/c) matings, where there is less than seven percent fetal loss [108]. The reason for fetal resorption in this mating combination is unknown, but is believed to represent immune mediated rejection of the semi-allogeneic fetus, because activated NK cells infiltrate the placenta and destroy the developing embryo. In addition, maternal immune cells from these matings respond to stimuli provided by placental antigens *in vitro* with production of the inflammatory cytokines: IFNγ, TNFα and IL-2 [109]. RNA levels of TNFα, IFNγ and IL-2 were significantly higher in placentas from mice of the abortion prone mating combination in comparison to normal matings [110]. Moreover, injections of anti-TNFα or anti-IFNγ antibody increased embryo survival [103] supporting the view that inflammatory cytokines are detrimental to pregnancy. CBA/J X DBA/2 placentas produce less anti-inflammatory IL-4 and IL-10 than to controls [104]. Embryonic rejection may also be directly related to the increased concentration of γδ T cells present at the fetal maternal interface in abortion-prone

females [111]. Addition of anti-γ chain antibodies resulted in reduced abortion rates in these animals [112]. More recently, in the murine model of RSA, abortions have been blocked effectively by antibodies to fgl2 prothrombinase, suggesting that rejection is mediated by a cytokine/ thrombocytic mechanism [113]. Deciduas in abortion prone pregnancies are characterized by an increased infiltration of maternal macrophages [114]. In an inflammatory immune environment, these cells may destroy fetal tissues through production of nitric oxide [115]. It is uncertain which cell type is responsible for the initiation of a TH1 immune response in the RSA mouse model but it is clear that maternal immune cells from abortion prone females are capable of produce cytokines that result in pregnancy termination.

In humans, at least half of the cases of spontaneous abortion can be attributed to factors that are genetic, hormonal, anatomical or infectious [116]. The remaining cases are proposed to result from maternal immune rejection of the fetus for unknown reasons [117]. A diagnosis of RSA syndrome or unexplained recurrent abortion (URA) reflects the clinical presentation of three or more successive miscarriages in the first trimester, in which none of the previously mentioned causative factors could be identified. RSA occurs in about 0.8 to 1% of pregnancies [118]. As in the mouse abortion-prone model, evidence suggests that inflammatory cytokines may play an important role in human spontaneous abortions. During normal human pregnancy, a decrease in the type 1 cytokines, IL-2 and IFN-γ, in serum has been observed [119]. In contrast, studies utilizing serum from women with URA found significantly higher concentrations of IFN γ, TNFα and TNFβ in comparison to controls [120]. Co-culturing of peripheral blood mononuclear cells (PBMC) from these women with autologous placental cells or antigens

from a trophoblast-derived cell line resulted in higher levels of IFNγ and relatively low levels of IL-6 and IL-10 production [121]. Immune system cells are highly prevalent in the pregnant uterus. Vince *et al* demonstrated that T cells and macrophages compose approximately 20% of decidual cells in the first trimester [89]. An increased number of activated T cells localizes to the decidua in women with RSA [122]. Another group specifically showed that T cells derived from RSA women exhibit a significant decrease in IL-4 and IL-10 production [123]. A subset of women with RSA was found to be deficient in critical TGF-β-secreting suppressor cells [124]. Furthermore, TNFα treatment of human trophoblastic cells results in apoptosis [125]. Collectively, these results indicate that TH1 immune responses are just as are detrimental to pregnancy in humans as they are in mice [126]. Interestingly, Arnold *et al* demonstrated that monocytes derived from RSA women showed a significant decrease in IL-10 expression level after treatment with PSG 11 [10].

Experimental evidence like that described above has led to the development of the theory that one important mechanism necessary to maintain the fetal allograft involves a shift in the maternal immune response during pregnancy towards a TH2-like environment [127]. This theory was postulated based on the levels of TH1 and TH2 cytokines in mice during pregnancy and their effects on the maternal immune system and the outcome of pregnancy. Anti-inflammatory cytokines are produced in part by the placenta, suggesting that the fetus itself participates in creation of the appropriate immune environment for successful pregnancy. For example, expression of mRNA for the anti-inflammatory cytokine IL-4 by murine placenta is 5-10 fold higher than in peripheral blood [128]. Murine placental tissues spontaneously secrete IL-4, IL-5 and IL-10, while maternal

lymph nodes and spleen do not [129]. Immunohistochemical staining of murine placental tissues (day 10.5) revealed that a large quantity of the TH2 cytokines, IL-4 and IL-10, are produced by trophoblasts [130]. In general, IL-10 is known to downregulate the production of inflammatory cytokines. There is evidence that TH2 immune responses can thwart abortive tendencies. In the mouse model of RSA, rejection can be prevented by treatment with exogenous IL-10, suggesting that attack on the fetus is mediated by the presence of TH1 cytokines [104]. Each of these examples demonstrates the importance of TH2 cytokines during murine pregnancy.

Less information is available about human cytokine production throughout pregnancy. Phytohaemagglutinin (PHA) stimulated PBMCs from pregnant women showed an increase in production of IL-4 and IL-10 [119]. As in the mouse, term human placental tissues secrete IL-10 [131, 132]. Furthermore, placenta from early elective abortions (weeks 8-10) and term placenta both stained positive by *in situ* hybridization for IL-10, IL-4 and IL-3 mRNA, suggesting placental secretion of these cytokines [99, 130]. It is intriguing to note that IL-10 mRNA is intensely localized in early invasive cytotrophoblasts yet it is only weakly present in these cells at term suggesting that IL-10 plays an essential role in pregnancy maintenance and possibly invasion but is repressed prior to parturition [130]. Co-culturing of normal maternal PBMC with placental antigens has demonstrated higher levels of IL-6 and IL-10 secretion than PBMC from women with RSA [121]. Therefore, secretion of anti-inflammatory cytokines is associated with successful pregnancy in humans.

Clinical correlation of the TH2 shift during pregnancy is derived from the effects of pregnancy on maternal autoimmune diseases (reviewed by [133, 134] and infectious

diseases. During pregnancy, diseases associated with cell-mediated pathologies such as rheumatoid arthritis, autoimmune thyroiditis and multiple sclerosis appear to follow a pattern of remission [135]. This effect is only temporary and symptoms return after the birth of the child. Conversely, Systemic Lupus Erythematosus (SLE), auto-immune thrombocytopenia (ATP) and, autoimmune hemolytic anemia, all humorally mediated autoimmune diseases, can be exacerbated during pregnancy [136]. Symptoms associated with infections by intracellular pathogens such as *Toxoplasmis gondii* and *Plasmodium malariae* are augmented during pregnancy. Normal resolution of these diseases occurs through activaty of the cell-mediated immune system [137]. Thus, the severity of autoimmune symptoms varies with the pregnancy-induced immune environment, and defects in the resolution of intracellular infections support the TH2-type shift paradigm.

Although both TH1 and TH2 cytokines are expressed over the course of pregnancy, the increased production of TH2 cytokines may counteract the potentially harmful effects of inflammatory cytokines at the fetoplacental junction and systemically through the innate immune response [126]. In addition, as stated by Wegmann "alternatively, a TH2 response leading to antibody production may be useful to the fetus since in many species these antibodies can be transferred across the placenta, resulting in protective immunity for the neonate before the endogenous immune system develops" [127]. The precise *in vivo* signaling molecules used to generate the TH2 bias are presently unclear. This bias may be brought by hormones such as progesterone, relaxin, cortisol and estrogen, and also by cytokines produced by macrophages, decidual cells, NK cells, T lymphocytes, and trophoblast cells [126, 127, 138-140]. Other proteins such

as PSGs, the focus of our studies, and growth factors like GM-CSF and CSF, may also contribute to the regulation of the immune response during pregnancy [11, 12, 141].

Other Potential Mechanisms of Pregnancy Maintenance

In addition to a TH2-like immune environment, other potential mechanisms involved in fetal protection include HLA-G expression, hormone secretion, indoleamine 2,3 dioxygenase (IDO) synthesis, CD95/CD95L mediated apoptosis, and reduced complement activation. HLA-G is a class I MHC molecule expressed on the surface of syncytiotrophoblasts in place of conventional MHC class I molecules [142]. In the absence of classical class I MHC molecules, such as HLA-A and HLA-B, at the fetal maternal interface, syncytiotrophoblast may be able to escape killing by NK cells via expression of HLA-G. Hormones, including progesterone and human placental growth hormone, have been reported to induce TH2 cytokine production and LIF expression [143]. Indoleamine 2,3 dioxygenase (IDO) has been shown to be essential for the success of murine pregnancy [144]. IDO is a tryptophan catabolizing enzyme that is expressed by macrophages and human syncytiotrophoblasts [145, 146], and is postulated to target metabolically active immune cells that are dependent on the availability of tryptophan. Pregnant mice treated with an IDO inhibitor displayed activated T cell rejection of the fetus. IDO production has also been reported in human dendritic cells [147] and murine endometrial cells during implantation [148], although it is unknown what triggers IDO production by these cells. CD95/CD95L (Fas and Fas ligand) initiates a well-studied apoptotic-signaling pathway. Tissue expression of Fas ligand (FasL) is associated with immune privileged sites, such as the eye and testis. It has been proposed that

trophoblasts express FasL, which could kill activated immune cells [149]. However experiments with genetically Fas-deficient mice revealed that this pathway may not be essential for fetal protection [130]. The importance of regulation of complement activation in protection of the murine fetus has been reviewed by Morgan and Holmes [150]. The role of complement during pregnancy was discovered using Crry knockout mice. Crry is a complement regulator in mice, and absence of this molecule resulted in embryonic death due to extensive C3 deposition in the placenta [151].

CD9 is a member of the Tetraspanin Superfamily

CD9 is a member of the tetraspanin superfamily of genes. Two independent groups identified the first tetraspanin (CD81) in 1990 [152, 153]. To date there are 24 members of the tetraspanin superfamily [154-156]. A brief list of closely related tetraspanin family members includes: CD9, CD37, CD53, CD63, CD81 and CD82. Some members of this family are expressed in a wide range of tissues (CD9, CD63, CD81, CD82), while others are cell-specific, like CD37, which is only expressed by mature B cells [154]. Tetraspanins are found in a variety of mammalian species and even on the surface of schistosomes (SM23)[152]. The diverse species expression of these proteins suggests that they play key biological roles, which have yet to be identified. Tetraspanin protein sequences predict structures with four hydrophobic transmembrane domains, one small and one large extracellular loop (EC1 and EC2) and short cytoplasmic domains at the C and N termini (figure 1).

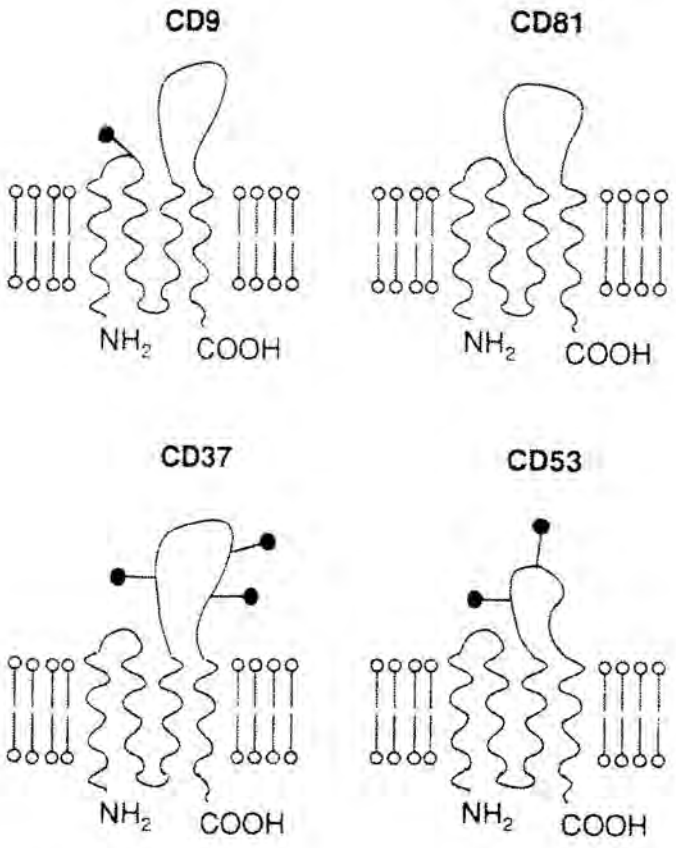


Figure 1. A schematic representation of the probable membrane orientation of four members of the transmembrane 4 superfamily (TM4SF). Potentially N-linked glycosylation sites are indicated by closed circles. (This figure was reproduced from Wright and Thompson. 1994. The Ins and Outs of the Tetraspanin Superfamily. *Immunology Today* **15** p.591.)

Amino acid sequence alignments of family members can be viewed on the tetraspanin homepage: www.ksu.edu/tetraspan/thepage.htm. (or [157]). There have not been any ligands identified for the tetraspanin superfamily.

Because of their potentially diverse roles in biological processes, tetraspanins have been described as "molecular facilitators"[154]. They mayfunction in regulating cell development, activation, growth, and motility [158, 159]. Without a known biological ligand for these proteins, much of the data regarding their function has been accumulated through use of anti-tetraspanin antibodies, comparison of tetraspanin expression in normal and cancerous tissues, and the coimmunoprecipatation of molecules that associate with tetraspanins on the cell surface. The latter has revealed a complex network of protein interactions between a number of tetraspanin family members and integrin molecules including $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_6\beta_1$ [160-164].

CD9 is a 24-27 kDa glycoprotein, which is widely expressed on hematopoietic and non-hematopoietic tissues [165, 166]. Sites of expression include platelets, monocytes, basophils, eosinophils, B cells, activated T cells, endothelial cells, neural cells, vascular smooth muscle, cardiac muscle and epithelia [167-174]. CD9 is also expressed by non-T acute lymphoblastic leukemia cells [175], and in 50% of acute myeloid and chronic lymphoid leukemia [165]. In both megakaryocytic and pre-B cell lines, CD9 is associated with the tetraspanins CD63, CD81 and CD82. Work by Rubinstein and Mannion showed that within this cluster, there is an association with the MHC class II molecule HLA-DR and the very late antigen integrins (VLAβ_1. 4 and VLA β_1-6) [161, 164]. The authors suggest that the networking of these cell surface molecules may indicate a role in signal transduction and cell motility, however the functional

significance of these interactions is still undefined. CD9 has also been found in association with the β2 integrin LFA-1 (CD11/CD18) [176].

A variety of antibodies to human and murine CD9 have been used to elucidate potential CD9 functions. Treatment with antibodies to CD9 have been shown to alter the differentiation of hematopoietic cells, block production of myeloid cells in long-term bone marrow cultures, and suppress proliferation of progenitor stem cells [177-179]. Some data obtained using anti-CD9 antibodies are contradictory. Anti-CD9 antibodies have been reported to induce proliferation of anti-CD3-activated murine T cells, followed by apoptosis due to insufficient IL-2 production by these cells [180]. In contrast, treatment of human CD9-transfected Jurkat cells with anti-CD9 and anti-CD3 antibodies resulted in an increase in proliferation and production of IL-2 [181]. CD9 expression in various cell lines has been associated with heightened or repressed chemotaxis [182, 183]. Identification of the epitopes on CD9 that are recognized by the different anti-CD9 antibodies, the CD9 binding partner(s), and the intracellular signaling pathways resulting in these biological effects may explain the diversity of reported functions.

Placental Implantation, Tetraspanins and Pregnancy

Immunohistochemical staining of CD9 revealed that it is differentially expressed on trophoblast cells [184]. Trophoblasts are specialized cells derived from the outer layer of the blastocyst. These cells are responsible for implantation into the uterine wall. Once partial implantation has begun, the trophoblast differentiates into two layers, an inner layer of mononuclear cells called cytotrophoblasts, and an outer syncytial layer called the syncytiotrophoblast. This layer is a true syncytium containing multiple nuclei and no

internal membranes to separate these organelles. In chorionic villi, the syncytiotrophoblast covers the cytotrophoblast cell layer (Figure 2). Cytotrophoblasts are the "stem cells" of placental development. They can develop into hormone-secreting villous syncytiotrophoblasts, extra-villous trophoblasts, or invasive intermediate trophoblasts. The hormones and proteins secreted by the syncytiotrophoblast layer, which are critical for pregnancy maintenance, include human chorionic gonadotropin, pregnancy specific glycoproteins, progesterone and estrogen. At the fetal-maternal interface, where the placenta comes in contact with uterine tissue, extravillous trophoblast develops into anchoring cell columns attached to both the placental villi and the uterine decidua. Invasive trophoblasts are responsible for restructuring extracellular matrix. These cells seek out maternal arteries, which they infiltrate, generating dilated vessels that will fill the intervillous space with blood from the mother. The maternal blood within the intervillous space provides for the exchange of nutrients, hormones and gases between the fetus and the mother. The pathological conditions pre-eclampsia, eclampsia and intra-uterine growth retardation are all associated with abnormalities in the trophoblast invasion process [64, 185].

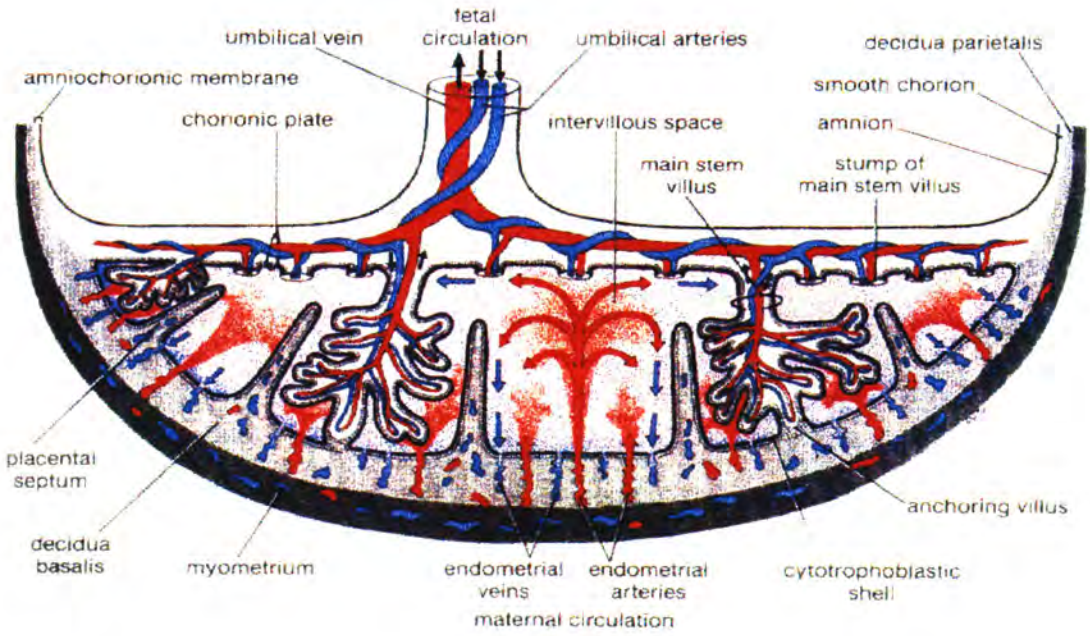


Figure 2. Schematic diagram of the human placenta. Three chorionic villi extending from the main stem villus are displayed in this schematic representation of the human placenta. The maternal portion of the placenta consists of the decidua basalis. The fetal portion consists of the chorionic plate, chorionic villi and the cytotrophoblast/ syncytiotrophoblast shell. Fetal vessels are lined by cytotrophoblasts which is directly covered by the syncytiotrophoblast layer. Maternal blood enter the intervillous spaces for the purpose of supplying the fetus with nutrients and oxygen and removing waste products. (Diagram reproduced from: Moore, KL. 1989. In Before We Are Born. Third Edition. Harvcourt Brace Jovanovich, Inc. PA, p. 92.)

Weak levels of CD9 are expressed on the surface of extravillous trophoblast cell columns and to a lesser degree on invasive trophoblasts [184]. Syncytiotrophoblasts do not stain positive for CD9 expression at 7 weeks post implantation. In the second and third trimester, CD9 is strongly expressed on extra-villous trophoblasts and chorion laeve, which is derived from extravillous trophoblast, but not on either cytotrophoblasts or syncytiotrophoblasts. Integrins α_3, α_5 and β_1 have also been identified on extravillous trophoblast [186]. Expression of CD9 appears to down-regulate the metastatic potential of these cells as it does in transfected melanoma cells [182] and highly metastatic cell lines [182]. These findings led to the hypothesis that fetal CD9 may have a regulatory role in the invasion of extravillous trophoblast at the maternal-fetal interface.

Using a human CD9 cDNA probe, murine CD9 was cloned and its amino acid sequence was predicted. Human and murine CD9 display 89% homology at the amino acid level [187]. To determine the physiological relevance of CD9, two groups independently generated CD9 knockout mice [188, 189]. Although both male and female CD9-/- mice appear biologically normal, female CD9-/- females exhibit a severe reduction in fertility. *In vitro* fertilization experiments revealed that sperm would not fuse with oocytes from CD9-/- females. CD9 is normally expressed on egg microvilli and becomes clustered at the site of sperm attachment along with integrin $\alpha 6\beta 1$ and fertilin [190]. Although the sperm attach normally, *in vitro* experiments demonstrated that fusion of egg and sperm did not occur [188, 190]. Interestingly, when wild type sperm were microinjected into CD9-/- egg cytoplasm, the number of successful offspring was equivalent to microinjection of CD9+/+ eggs [189]. Mating between male CD9-/-

mice and wild type or heterozygous females resulted in normal levels of fertilization, further indicating the necessity of CD9 on the oocytes during fertilization [188].

Specific Aims:

1. To study cytokine expression of macrophages treated with the N-domain of PSG17.
2. To characterize PSG-macrophage binding
3. To clone the murine PSG Receptor

Significance:

The mechanisms, which generate maternal tolerance to the semi-allogeneic fetus are intertwined and essential for fetal survival. Human PSG secretion begins within days of conception and increases throughout pregnancy. Reduced concentrations of human PSGs are prescient of spontaneous abortions. Human PSGs have been shown to increase cytokine production in macrophages. Each of these factors suggests that PSGs may play a role in the network of fetal protection from maternal immunological response. This study was designed to further investigate PSG immuno-regulatory mechanisms and develop a mouse model of PSG function, including identification of the PSG receptor.

Hypothesis and Approach:

With the knowledge that murine PSG18 and human PSGs: PSG1, PSG6 and PSG11 induce cytokines in macrophages, we will investigate the effects of the N-domain of PSG17 on a murine macrophage cell line and in thioglycollate-induced peritoneal macrophages from BALB/c mice. Based on the highly homologous nucleotide and amino acid sequences of this family and the similar biological effects observed with

human PSGs, we propose that murine PSG17N will induce cytokine responses in these cells.

Cloning of the PSG receptor will allow us to identify other biological functions for these proteins, and to ascertain whether these proteins share a common receptor. Before choosing the cloning procedure to isolate the putative receptor, we sought to biochemically characterize the PSG-receptor cell surface binding interactions and identify the molecular weight of the interactive molecule(s). To determine the affinity of the PSG ligand for its putative receptor, binding curves for murine macrophages will be generated utilizing recombinant PSG-alkaline phosphatase fusion proteins. To characterize the receptor(s) molecular weight, biotinylated macrophage cell membranes will be poured over PSG coated Nickel NTA beads (Qiagen). The protein(s) isolated by the ligand-coated beads will be separated on an SDS-PAGE gel, transferred to nitrocellulose and identified with streptavidin-HRP. The protein(s) molecular weight will be determined based on standard molecular weight markers.

Results:

PSG17N induced expression of IL-10, IL-6 and TGF-β1 in both RAW 264.7 cell and peritoneal macrophages. The concentrations of PSG17N required to induce these cytokines in RAW 264.7 cells were significantly less than that of PSG18N. In addition, we determined that the PSG17N increase of IL-10 and IL-6 mRNA in these cells required de novo protein synthesis. Characterization of the PSG-macrophage cell surface binding interactions revealed that there are approximately 1770 PSG17 binding sites per RAW cell with a disassociation constant of 2.2×10^{-11} M. Based on the ability of PSG17N to

induce cytokines in RAW cells and the high affinity of the PSG17 RAW cell binding interaction, these cells were chosen as the RNA source to construct the cDNA expression library for the purpose of cloning the receptor. Panning on PSG17N coated dishes was utilized to screen the expression library. In the work described here, it was determined that CD9 is the receptor for murine PSG17. In elucidating the PSG17-CD9 interaction, we also discovered the first biological ligand identified for any member of the tetraspanin superfamily. Therefore, CD9 appears to have more than one important function in murine pregnancy: CD9 is important for egg-sperm fusion, it may regulate invasion into the maternal tissues, and as a receptor for at least one PSG, it may influence the maternal immune system.

II. Paper #1

Pregnancy Specific Glycoprotein 17 Binds with High Affinity to Murine Macrophages and Induces Production of Cytokines *in vitro*.

Waterhouse, R., Wessells, J., White, K., Rollwagen, F., Zimmermann, W., and Dveksler, G.

ABSTRACT

Pregnancy specific glycoproteins (PSGs) are a family of secreted proteins produced by the placenta throughout pregnancy. In human serum, the concentration of these glycoproteins thoroughly exceeds that of other pregnancy related proteins, reaching up to 200-400 μg/ml at term. Despite their high concentration in maternal serum, no *in vivo* biological functions for these proteins have been identified. In recent reports, we have shown that treatment of cultured human elutriated monocytes with three recombinant human PSGs induced IL-10, IL-6 and TGF-β_1 secretion. In addition, murine PSG18 was shown to increase IL-10 mRNA and protein expression in murine macrophages, but its effects on IL-6 and TGF-β_1 secretion were not investigated. To determine whether murine PSG family members have overlapping functions and mimic human PSG biological effects, RAW 264.7 cells and thioglycollate-induced peritoneal macrophages were treated with murine PSG17. We examined the levels of IL-10, IL-6 and TGF-β_1 secretion by ELISA after treatment with PSG17N a recombinant protein consisting of the N- domain of PSG17. PSG17N significantly induced secretion of all three cytokines. Treatment of RAW 264.7 cells with PSG17N in the presence of cycloheximide significantly reduced IL-10 and IL-6 mRNA levels indicating production of these cytokines required de novo protein synthesis. Additional characterization of the PSG-RAW 264.7 cell interaction was pursued with a recombinant PSG17-alkaline

phosphatase fusion protein. Scatchard analysis revealed there were approximately 1770 binding sites per cell with a K_D of 2.2 X 10^{-11} M. The overlapping functions of various PSGs and their high affinity to a ligand present on macrophages support the hypothesis that these placentally secreted glycoproteins are involved in the regulation of the maternal immune response.

INTRODUCTION

Pregnancy specific glycoproteins (PSGs) are a family of highly homologous proteins secreted by the placenta. They were originally isolated from the circulation of pregnant women (1). PSGs are detected in maternal blood as early as seven days post implantation. The serum levels of these proteins reach up to 200-400 μg/ml at term, far exceeding the concentration of human chorionic gonadotropin and alpha fetoprotein (2). Abnormally low levels of PSGs are associated with several serious complications of pregnancy including fetal hypoxia, fetal growth retardation, pre-eclampsia and spontaneous abortion (3-6). PSG homologues have been identified in mice, rats, monkeys, and most recently baboon (7-9). Treatment with anti-PSG antibodies in two animal models resulted in spontaneous abortion (10, 11). The high concentration of PSGs in maternal serum and, their linkage to fetal pathologies and experimentally induced abortions indicate that PSGs play a key role in pregnancy.

There are 11 human PSG genes (*PSG1-PSG11*) localized to chromosome 19. Alternative splicing of these genes results in more than 37 different gene products. Transcripts from these genes are co-expressed during pregnancy. A peptide derived from the N-terminal domain of human PSG11 was shown to bind to macrophages, but not to B

or T cells (12). Studies with recombinant PSGs revealed that PSG1, PSG6, and PSG11 induced the production of interleukin 10 (IL-10), IL-6 and transforming growth factor β_1 ($TGF\beta_1$) by human monocytes (13). Interestingly, activated monocytes from women with recurrent spontaneous abortion displayed a decrease in PSG11-induced expression of IL-10 (14). Together, these findings suggest that human PSGs are immune regulatory factors, which can potentially affect maternal immune responses during pregnancy.

Partial genomic sequences have revealed that there are 14 murine PSG genes (*PSG16-29*) localized to chromosome 7, which is syntenic to human chromosome 19 (8). Murine PSGs are similar to human PSGs in that they both contain several immunoglobulin-like domains and are heavily glycosylated. In both species the N-domain is immunoglobulin-variable like. The amino acid sequences in this region are highly conserved between PSG genes within each species (54-94% in murine PSGs). To date, full-length cDNAs have been reported for PSG17, 18 and 19 (formerly *Cea2, 3, 4*) (8, 15). Murine PSG18 induced an increase in expression of IL-10 mRNA and protein in a macrophage cell line and peritoneal macrophages (15). Whether murine PSG17 and PSG19 also induce IL-10 had not been reported.

In this study, we investigated the induction of cytokine secretion by murine PSG17 using a murine macrophage cell line and thioglycollate-induced peritoneal macrophages. We also examined the binding kinetics of the PSG-macrophage interaction. Because murine PSG17 induced IL-10, IL-6 and $TGF\beta_1$ in murine macrophages, we propose that murine PSGs have biological functions similar to human PSGs. In addition, characterization of PSG-macrophage binding revealed that PSG17 binds with high affinity to a putative receptor on the surface of these cells. Together,

these results support a role for PSGs as immune regulatory molecules that could potentially influence maternal innate immune responses and fetal tolerance.

MATERIALS AND METHODS

Animals and Cell Culture

RAW 264.7 cells (an Abelson leukemia virus-transformed macrophage cell line of BALB/c origin) were obtained from American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, 5mM sodium pyruvate (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (PSA) and 10% fetal bovine serum (FBS). HEK 293T (PEAK^{Rapid}) cells (Edge BioSystems, Gaithersburg, MD) were cultured in DMEM, 10% FBS, 50 μg/ml gentamicin (Quality Biological Inc, Gaithersburg, MD), 250 μg/ml G418 (CalBiochem, La Jolla, CA) and PSA. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Five to six week old BALB/c mice were purchased from the NCI laboratories (Fredrick, MD). The animals were placed in cages with filter tops and fed standard chow and water ad libitum. Peritoneal macrophages were harvested and cultured as previously described (15).

Reagents

To generate recombinant PSG17N-Myc-His proteins, the N terminal domain of PSG17 was amplified by PCR from full length PSG17 (16) in pBluescript II KS+ (Stratagene, La Jolla, CA) using the oligonucleotide primers 5' GAAGATCTAGAG

ATATGGAG(T/G)TGTC3' (underlined *Bgl*II site) and 5' TTGGTACCCTCATTT (A/G)TCACAG(C/T) CAGG 3' (underlined *Kpn* I site). The PCR product was inserted into the pCRII-TOPO cloning vector (Invitrogen, Carlsbad, CA). To add the myc-epitope and 6X histidine tags to the N terminus of the cDNA, PSG17N was removed from pCRII-TOPO by digestion with *Bgl*II and *Kpn*I, and ligated into pcDNA3.1 Myc-His (Invitrogen). PSG17N-Myc-His was inserted into the *Not*I and *Pst*I digested pFastBac (Life Technologies, Rockville MD). Recombinant baculovirus was obtained following manufacturer's instructions (Life Technologies, Rockville, MD) and purified from insect cell supernatant as previously described for PSG18N-Myc-His (15)[1]. The GST-His-XylE control protein was purified as described (15) using glutathione-sepharose beads. All of the recombinant proteins were tested for endotoxin contamination using the *Limulus* amebocyte lysate assay (Biowhittaker Inc., Woburn, MA). Only endotoxin free recombinant proteins were used in experiments measuring cytokine production.

Subcloning of PSG17FL into Alkaline Phosphatase (AP)-Tag4

To generate AP-PSG17, full length PSG17 was amplified from PSG17-pBluescript II KS using the sense primer 5' GAAGATCTAGAGTCACTGTGGAATT 3'(underlined *Bgl*II site) and the anti-sense primer 5' TCACACGATCATCACAGCCAG 3', and cloned into PCR Script KS+ (Stratagene). The cDNA was excised with *Bgl*II and *Xho*I and inserted into complementary sites within the AP-Tag4 vector (generously supplied by Dr. John Flanagan, Harvard University), which resulted in a fusion protein composed of the secretion signal followed by an alkaline phosphatase and the PSG17 coding sequence at the C-terminus. Protocols for generation and quantitation of heat

[1] Thesis Footnote: Refer to Appendix 2 for a detailed explanation of PSG17NMyc-His purification scheme.

stable AP-fusion proteins have been reported by Flanagan and Cheng (17). HEK 293T cells were transfected with the PSG17-APTag4 vector using Lipofectamine PLUS (Life Technologies). Cell supernatants containing the AP proteins were harvested 5 days post-transfection, filtered through a 22 micron filter, and 10 mM HEPES and 0.1% sodium azide were added. The supernatants were heated to 65°C for 10 minutes to inactivate endogenous phosphatases. Serial dilutions of harvested supernatants were mixed at a 1:1 ratio with 2X AP substrate (12 mM p-nitrophenyl phosphate (Sigma Chemicals, St. Louis, MO), 0.5 mM $MgCl_2$, 1 M diethanolamine [pH 9.8], 0.5 mg/ml bovine serum albumin, and incubated for 1 hour at room temperature. The concentration of AP protein was measured by de-phosphorylation of p-nitrophenyl phosphate which was quantitated by absorption at 405 nm using an ELISA plate reader. When high concentrations of fusion protein were necessary, supernatants were concentrated in a centriprep-10 and re-assayed before treating cells (Millipore, Bedford, MA). Dilutions of AP-proteins for binding assays were made in HBHA buffer (Hank's balanced salt solution; 0.5 mg/ml BSA, 20 mM HEPES [pH 7.0], 0.1% NaN_3).

AP-Fusion Protein Binding Assays

RAW 264.7 cells were grown to confluence in a 6-well plate and washed with cold HBHA buffer. AP-PSG17 or the AP control protein was added to the cells in triplicate wells at a designated concentration. After 90 minutes at 4°C, the proteins were removed, and the cells were washed seven times with HBHA buffer. Cell lysates were harvested with lysis buffer (1% Triton X-100, 10mM Tris –HCl [pH 8.0]), thoroughly vortexed, and then centrifuged to pellet nuclei and debris. The cleared lysates were

transferred to clean tubes and heated to 65°C for 10 minutes. The concentration of AP and AP-PSG17 proteins in the cleared lysates were quantitated by enzymatic assay as indicated above.

Analysis of Cytokine induction by PSG-treated macrophages and RAW 264.7 cells

RAW 264.7 cells and peritoneal macrophages were seeded in 24 well tissue culture plates at 1 X 10^6 or 1.5 X 10^6 cells per well, respectively. The cells were treated in triplicate with PSG17N-Myc-His for 4 hours at 37°C in a volume of 300 µl. After 4 hours, the volume was increased to 1 ml in each well by addition of cell culture media. Supernatants were harvested at 2, 6, or 24 hours post-treatment as indicated. Secreted IL-10, TGF-β_1, PGE_2 and IL-6 were measured by ELISA (R & D Systems, Minneapolis, MN) (Pierce-Endogen, Woburn, MA). The limit of detection of the IL-10, IL-6 and TGFβ_1 ELISA assay was 7 pg/ml and the PGE_2 assay was 36.2 pg/ml. Prior to measuring IL-10 and IL-6 secretion by BALB/c peritoneal macrophages, the supernatant was concentrated 2.5 fold with a microcon -YM3 concentrator (Millipore). Treatment with LPS was used as a positive control for these experiments.

Semi-quantitative RT-PCR

RAW cells were grown in a 24 well plate were treated with 15 µg/ml recombinant PSG protein or the control protein GST-His-XylE and cycloheximide (Sigma) was added at a concentration of 5 µg/ml where indicated. Total RNA was harvested two hours post-treatment using TRIzol (Life Technologies) according to manufacturer's instructions. RNA was reverse transcribed using random hexamers and

Ready-to-go you-prime-first strand beads (Amersham Pharmacia, Piscataway, NJ). One tenth of the reverse transcriptase reaction was used for PCR amplifications of IL-10, IL-6, or GADPH. Various PCR cycles were analyzed to optimize the linear correlation between RNA and PCR product. Amplified products were electrophoresed on a 1.5 % TBE agarose gel and blotted onto Nytran membranes. The membranes were UV cross-linked, baked for 30 minutes at 80°C, and then hybridized with an internal ^{32}P- labeled oligonucleotide probe. After hybridization, the band intensity was quantified using the Storm Phosphorimager and Image QuaNT program (Molecular Dynamics, Sunnyvale, CA). IL-10 and IL-6 cDNA intensity measurements were normalized to the GADPH PCR product value (18).

Data Analysis

Data was obtained from at least three independent experiments. Results were evaluated for statistical significance using the unpaired student's *t* test. Data was expressed as mean ± standard error (S.E.) and significance was defined at $p < 0.05$.

RESULTS

PSG17 induces secretion of IL-10 in murine macrophages in a dose dependent manner

We previously reported that 20-25 μg/ml murine PSG18N was required to induce IL-10 in RAW 264.7 cells (15). To determine if murine PSG17 and PSG18 have similar biological effects, RAW 264.7 cells were treated with increasing concentrations of truncated recombinant PSG17 protein consisting of the N-domain (PSG17N-Myc-His) or a control protein GST-His-XylE, and IL-10 secretion was measured six hour post-

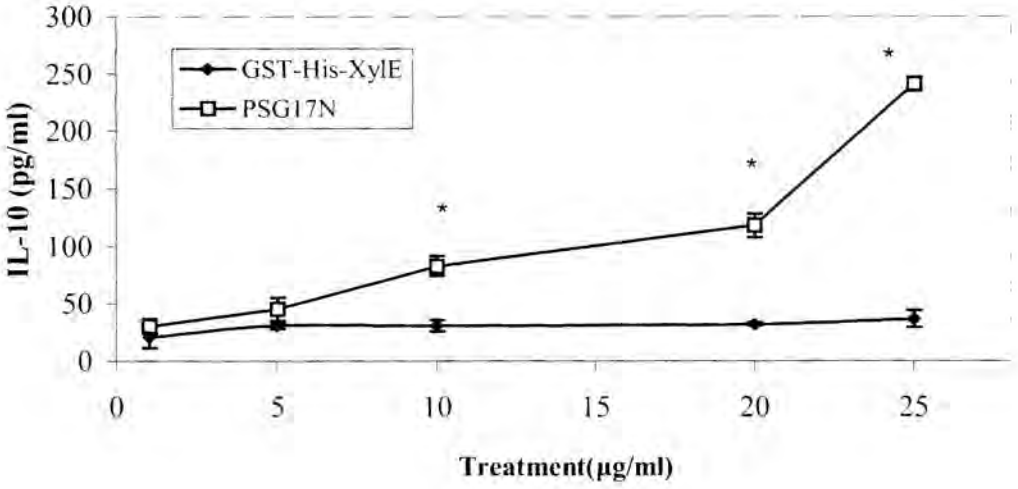
treatment. Immunoreactive IL-10 showed a dose dependent increase in secretion after treatment with PSG 17N (Figure 1A). PSG17N induced significant levels of cytokine secretion at a concentration as low as 10 μg/ml. A time course analysis revealed that PSG17N treatment of RAW 264.7 cells resulted in an up-regulation of IL-10 mRNA expression within two hours post treatment (data not shown). Based on these data, we investigated the ability of PSG17N to induce IL-10 in thioglycollate-induced primary peritoneal macrophages. Treatment with PSG17N at a concentration of 25 μg/ml resulted in significantly increased secretion of IL-10 in comparison to control protein and untreated cells (figure 1B). The amount of IL-10 induced by PSG17N in peritoneal macrophages was less than the amount induced by the positive control, 100 ng/ml LPS (data not shown).

PSGs induce production of IL-6 and TGFβ1 in murine macrophages

Previously, we showed that recombinant human PSG1, PSG6 and PSG11 induced secretion of IL-10, IL-6 and $TGF\beta_1$ by human elutriated monocytes (13). To determine if murine PSG17 treatment would mimic these effects in murine macrophages, we examined levels of IL-6 and $TGF\beta_1$ secretion by RAW cells, six hours after treatment. PSG17N induced significant amounts of IL-6 at a concentration of 10 μg/ml, and the response was dose dependent (Figure 2A). IL-10 and IL-6 secretion was observed only after treatment of RAW cells with murine PSG18N[2] at concentrations higher than 20 μg/ml (data not shown). In addition, peritoneal macrophages treated with 25 μg/ml

[2] To be included only in Thesis publication: PSG18N induction of IL-6 was performed by Jennifer Wessells as part of her graduate thesis work (1999) at the Uniformed Services University of the Health Sciences entitled "The Cloning, Characterization and Functional Analysis of Murine Pregnancy Specific Glycoproteins." It is presented in this manuscript as a contributing data point to the whole and should be so noted.

A.



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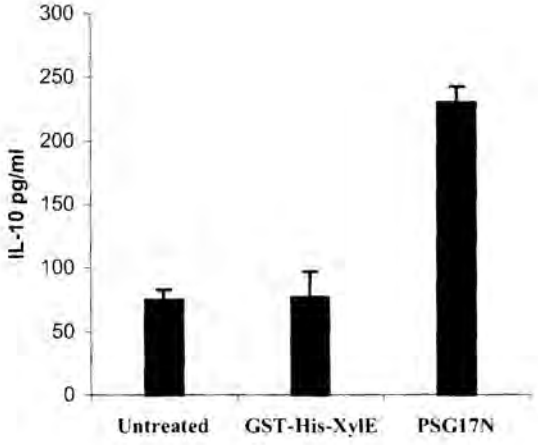
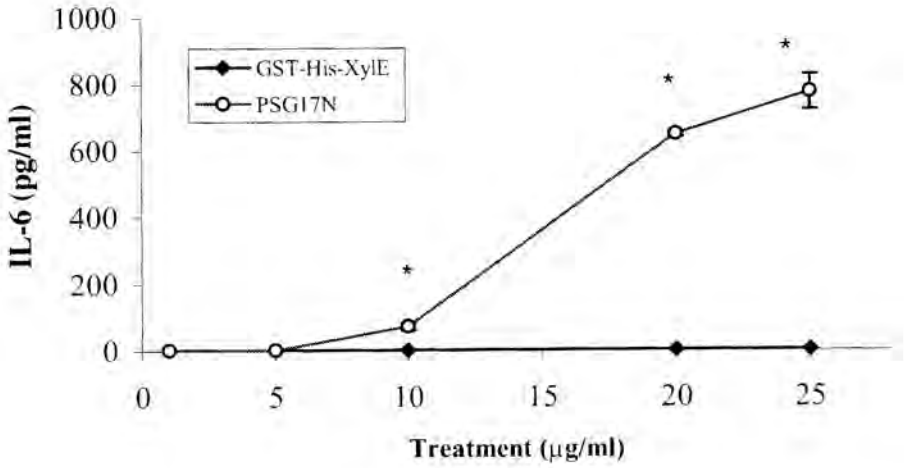


Figure 1. PSG17N induces IL-10 secretion in murine macrophages and RAW 264.7 cells. (A) RAW 264.7 cells were treated in triplicate with increasing doses of PSG17N-Myc-His or the control protein GST-His-XylE. Cell supernatants were harvested six hours post-treatment, and IL-10 secretion was measured by ELISA. All data shown are representative of at least three independent experiments (*$p<0.05$). (B) BALB/c peritoneal macrophages were treated in triplicate with 25 µg/ml PSG17N-Myc-His or GST-His-XylE, and IL-10 production was measured from concentrated supernatants by ELISA.

A



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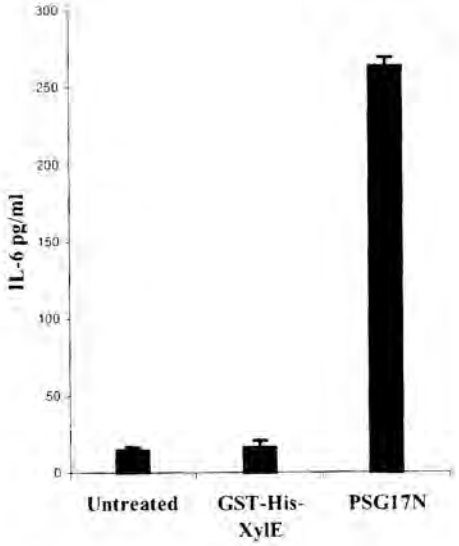


Figure 2. PSG17N induces IL-6 secretion in murine macrophage and RAW 264.7 cells. (A) RAW cells were treated in triplicate with increasing doses of PSG17N-Myc-His or GST-His-XylE. Supernatants were harvested 6 hours post-treatment, and IL-6 secretion was assayed by ELISA. (B) Thioglycollate-induced peritoneal macrophage from BALB/c mice were treated in triplicate with PSG17N or GST-XylE-His at 25 μg/ml. All data are representative of three experiments (*p<0.05).

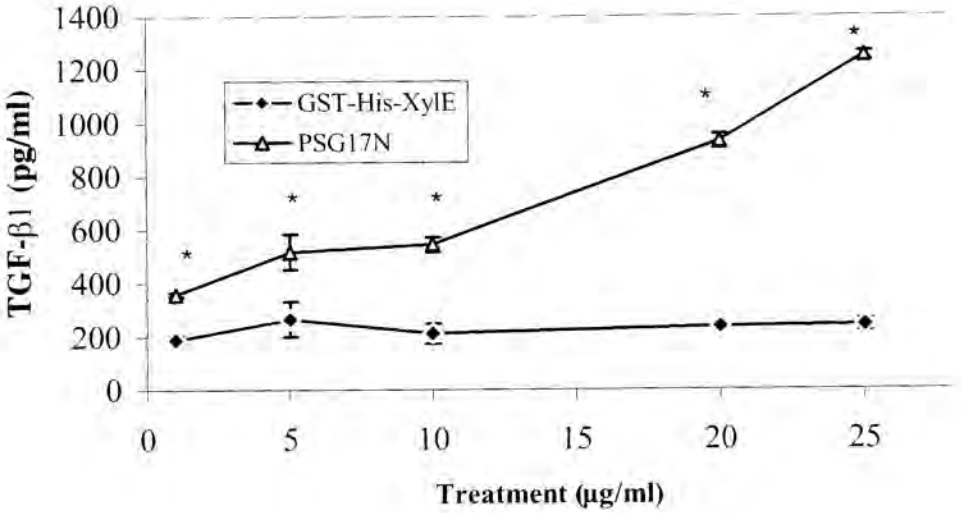
PSG17N showed a significant increase in IL-6 secretion (Figure 2B). PSG18N also induced IL-6 secretion in BALB/c peritoneal macrophage at 25 μg/ml (data not shown).

TGFβ$_1$ is associated with increased production of IL-10 and is believed to be an essential immuno-regulatory cytokine during pregnancy (19). In RAW cells, PSG17N induced TGFβ$_1$ secretion in a dose dependent manner (Figure 3A), and significantly increased TGFβ$_1$ production in BALB/c peritoneal macrophage at treatments of 10 μg/ml (data not shown) and 25 μg/ml (Figure 3B). Unlike what was observed in RAW cells, PSG17N induced significant levels of TGFβ$_1$ protein as early as two hours post treatment in peritoneal macrophages, but not at 24 hours (data not shown).

PSG induction of IL-6 and IL-10 requires de novo protein synthesis

Since delayed induction of IL-10 and IL-6 (twelve hours and six hours, respectively) is observed after PSG treatment of human monocytes, we proposed that de novo protein synthesis is required for PSG-induced IL-10 and IL-6 up-regulation (13). To evaluate this hypothesis, RAW cells were treated with PSG17N in the presence or absence of the translational inhibitor cycloheximide. In the presence of cycloheximide, there was a significant decrease in IL-10 and IL-6 mRNA compared to treatment in the absence of cycloheximide (Figure 4). Co-treatments of LPS and cycloheximide resulted in up-regulation of IL-10 mRNA in comparison to treatment with LPS alone (data not shown).

A



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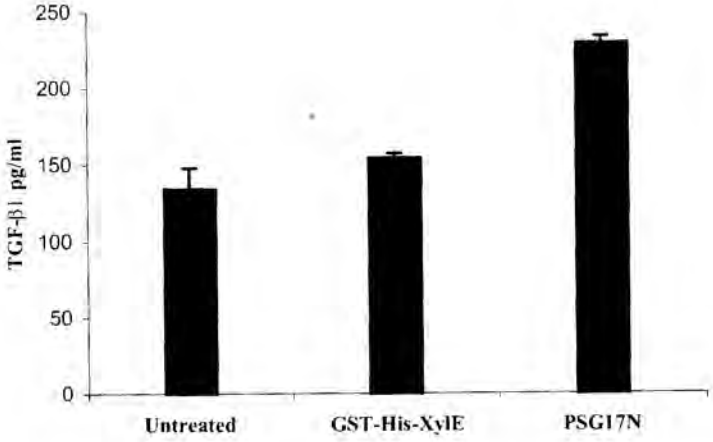


Figure 3. PSG17N induces TGFβ_1 secretion by murine macrophage and RAW 264.7 cells. (A) RAW 264.7 cells were treated with increasing doses of PSG17N-Myc-His or GST-His-XylE. At 24 hours post-treatment TGFβ_1 was assayed by ELISA. Data is representative of four experiments (*p<0.05). (B) BALB/c thioglycollate-induced peritoneal macrophages were treated with PSG17N 25 µg/ml. TGFβ_1 was measured by ELISA in concentrated supernatants from macrophages 2 hours post-treatment. Data is representative of three independent experiments.

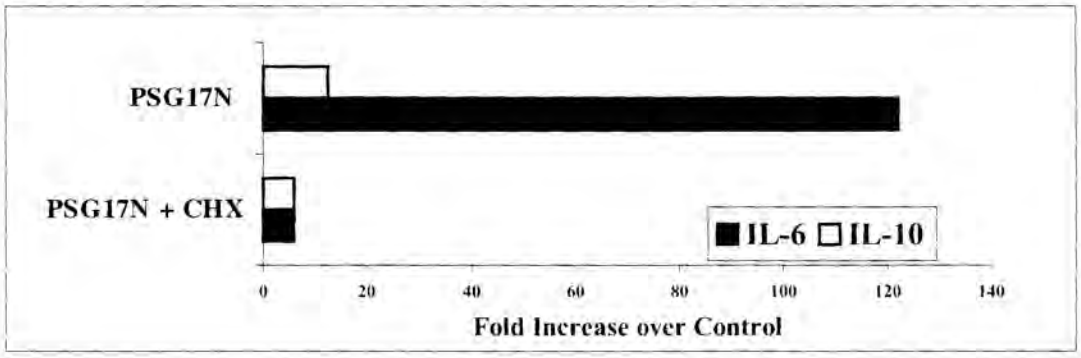


Figure 4. Cycloheximide inhibits PSG17N induction of IL-6 and IL-10 mRNA. RAW 264.7 cells were treated with 10 μg/ml PSG17N-Myc-His or GST-His-XylE in the presence or absence of 5 μg/ml cycloheximide (CHX). RNA was harvested at 2 hours for IL-10 and at 4 hours for IL-6 and levels of IL-10, IL-6 and GADPH were determined by RT-PCR. The values reported were normalized to the GADPH values for each treatment and subsequently divided by the values for the GST-His-XylE control treatment.

PSG17 induces the eicosanoid, Prostaglandin E_2 in RAW 264.7 cells

Prostaglandins are produced in low levels throughout pregnancy and at high levels during labor (20) and PGE_2 can regulate production of IL-10 and IL-6 in murine macrophages (21). Therefore, we decided to test for the presence of signaling this molecule in RAW cell supernatants after treatment with 10 μg/ml PSG17N. Significant levels of this eicosanoid were produced in response to PSG17N (Figure 5). Supernatant analyzed at two, four and six hours post treatment showed that PGE_2 secretion peaked at two hours. Expression was not significant at 24 hours post treatment (data not shown). In contrast, thioglycollate induced peritoneal macrophages did not demonstrate a significant increase in PGE_2 secretion at two hours post treatment (data not shown).

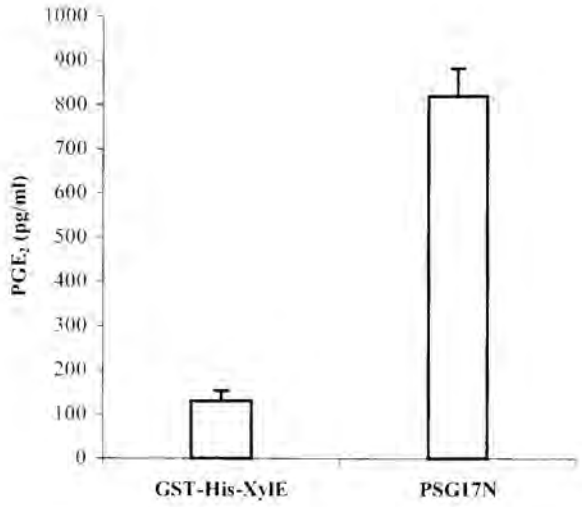
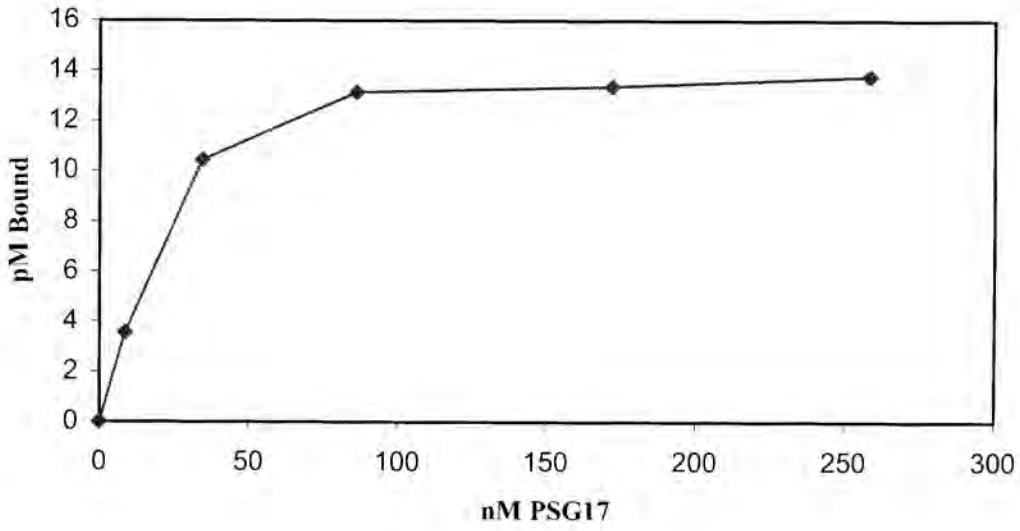


Figure 5. PSG17N induced PGE_2 in RAW 264.7 cells. RAW cells were treated in triplicate with 10 μg/ml PSG17N-Myc-His or the control protein GST-His-XylE. Supernatants were harvested 2 hours post-treatment and the concentration of PGE_2 was determined by ELISA.

PSG17 binds with high affinity to a limited number of binding sites on RAW 264.7 cells

Induction of cytokine and prostaglandin secretion in macrophages by PSGs, implies the existence of a macrophage-PSG receptor. To characterize the binding of PSG17 to RAW cells, we cloned the full-length PSG17 cDNA into a vector containing an alkaline phosphatase (AP) tag. RAW cells were treated in triplicate with increasing concentrations of AP-PSG17 fusion protein or AP only. Saturation of PSG17 binding sites was observed at 86 nM (Figure 6A). Conversion of the binding data to a Scatchard plot revealed that there are an average of 1770 binding sites per RAW cell for PSG17 (Figure 6B) with a K_D of approximately 2.2×10^{-11} M (Figure 6B).

A.



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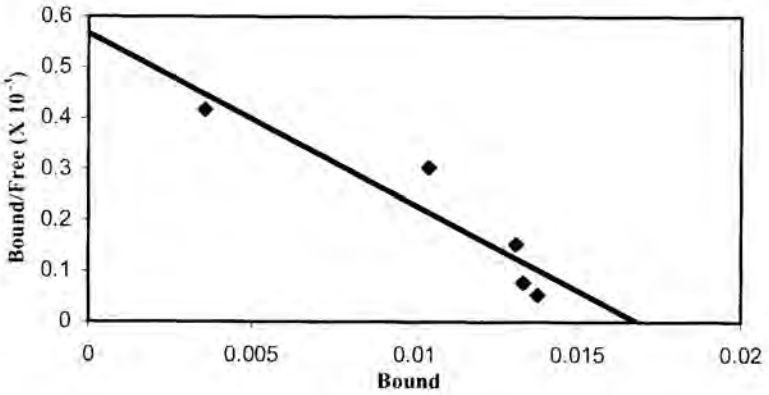

Figure 6. Scatchard analysis of PSG17 binding to RAW 264.7 cells. (A) RAW 264.7 cells were treated in triplicate with increasing concentration of AP-PSG17 or the AP control. PSG17 binding values were plotted after subtracting levels of non-specific binding by AP from total binding. Similar binding curves were obtained in three independent experiments. (B) Scatchard analysis of binding revealed an average of 1770 binding sites per cell with K_D of approximately 2.2 $X10^{-11}$ M.

DISCUSSION

Beginning at conception, the fetus is a chimera of both paternal and maternal antigens. Although the maternal immune system may recognize the presence of the semi-allogeneic fetus, in most cases, a detrimental immune response does not ensue. For this reason, pregnancy is a mysterious immunological phenomenon. Based on experimental evidence, various mechanisms, that are not mutually exclusive, are potentially responsible for fetal tolerance (reviewed by Thellin *et al.*) (22). The most relevant, with respect to the data we present here, is the development of a bias toward a TH2 or anti-inflammatory maternal immune environment of the maternal immune response during pregnancy (23). A TH2 immune environment is characterized by the presence of specific cytokines including IL-10, TGF-β_1 and IL-4. These cytokines reduce the expression of pro-inflammatory cytokines, which are known to be detrimental to pregnancy (24).

Macrophages are an essential source of cytokines during innate immune responses. These cells are affected by various circulating factors produced during pregnancy (25). In this study, murine macrophages were treated with one such factor PSG17, that is normally secreted by the placenta. Using recombinant PSG proteins, we examined macrophage secretion in response to PSG17, as well as cell binding parameters. We ascertained that murine PSG17 binds with high affinity to a putative receptor on the murine macrophage cell line RAW 264.7. We also showed that the *in vitro* biological effects of two murine PSGs are similar. Both murine PSG17 and PSG18 induce secretion of IL-10 and IL-6, albeit at different concentrations. Regulation of cytokine secretion by macrophages may be a critical function of these proteins as both murine and human PSGs induced IL-10, IL-6 and TGFβ_1.

Through induction of IL-10, PSGs could contribute to the down-regulation of inflammatory TH1 cytokines locally at the fetal maternal junction and/or systemically as these PSGs circulate in maternal blood. IL-10 was essential in reducing inflammatory responses in animal models (26, 27). Increased concentrations of IL-10 have been reported at the fetal-maternal interface in normal pregnancies in mice and humans (28). Decreased concentrations of IL-10 in serum have been associated with preeclampsia and spontaneous abortion (14, 29)((30). A correlation between decreased endometrial production of human PSG11 transcripts and recurrent spontaneous abortion in women has also been reported (14).

IL-6 secretion was induced by macrophages using similar concentrations of PSG17N. IL-6 is a multi-functional cytokine with the ability to stimulate a variety of different cells. IL-6 treatment induces T and B cell growth and differentiation, and enhances the differentiation of hematopoietic precursors. In association with TNFα and IL-1, IL-6 augments acute phase protein synthesis in hepatocytes, but it has also been demonstrated to act as an important anti-inflammatory cytokine both locally and systemically (31). Uterine decidual cells and macrophages have been reported to express IL-6 during implantation and throughout pregnancy in mice (32-34). Production of this cytokine inhibited the IL-1 and TNFα production by macrophages (35). Based on these reports, PSG induction of IL-6 by macrophages may also play important roles in placental regulation of maternal immune responses and in placental development. For example, IL-6 constitutively secreted by trophoblast cells has a positive feedback effect on IL-6 receptor expression, and induces production of human chorionic gonadotropin (HCG) from first trimester human trophoblasts in culture (36).

In our experiments, TGFβ_1 secretion was up-regulated within 2 hours of PSG17 treatment in thioglycollate-induced macrophages, however, significant induction of this cytokine was not detected at 24 hours in RAW cells. The different kinetics in TGFβ_1 induction in thioglycollate-elicited macrophages and the murine macrophage cell line may reflect the differing activation states of the cells. TGFβ_1 increases IL-6 production in peripheral blood mononuclear cells, and up-regulates IL-10 production in murine macrophages, which may explain the significance of early production of this cytokine by activated macrophages (37). The relevance of PSG-induced TGFβ_1 secretion by macrophages to the later IL-10 and IL-6 up-regulation remains to be determined. In contrast to IL-6 and IL-10 induction, as little as 1 μg/ml of PSG17 was necessary to enhance the levels of TGFβ_1. Human PSG1, PSG6 and PSG11 also induced significant levels of IL-6, IL-10 and TGFβ_1 at 24 hours post-treatment in murine RAW cells (13). Together, this data indicates that certain murine and human PSGs may have similar biological functions.

Secretion of IL-6 and IL-10 by RAW cells after treatment with PSG17 required de novo protein synthesis. The proteins required to be synthesized prior to IL-10 and IL-6 mRNA up-regulation are presently unknown. In an attempt to identify potential inducers of these cytokines, we analyzed expression of the eicosanoid, PGE_2 and the pro-inflammatory cytokine TNFα (data not shown). PSG17N induced PGE_2 in RAW cells within 2 hours of treatment. Interestingly, PGE_2 regulates IL-6 and IL-10 production by activated macrophages (21). Secretion of PGE_2 has also been described in uterine macrophages during pregnancy. PGE_2 has been reported to regulate T cell differentiation, favoring TH2 T-cell development through induction of IL-10 (38). The

levels of PGE_2 measured at 6 hours post-PSG17N treatment were similar to the values obtained after treatment with the control protein (data not shown). This down-regulation of expression at 6 hours might be explained by the presence of IL-10 in the media. IL-10 has been shown to provide negative feedback for PGE_2 secretion in macrophage and placental cells (39, 40). Recently, experiments with spleen cells from IL-10 knockout mice revealed that endogenous IL-10 is a regulator of LPS induced PGE_2 synthesis *in vivo* (41). To confirm that IL-10 secretion was not a result of earlier macrophage production of pro-inflammatory cytokines, we tested supernatants from PSG17N-treated macrophages for the presence of TNF-α at two hours post-treatment. PSG17 did not increase production of this cytokine. This concurs with our previous data from macrophage treatments with human PSG 1, 6 and 11 and murine PSG18, none of which increased secretion of inflammatory cytokines at the RNA or protein level. The absence of TH1 type cytokines in response to PSGs suggests that these proteins are not non-specifically activating macrophages, but are inducing a specific response (unlike LPS, which is a potent inducer of both TH1 and TH2 cytokines in macrophages.) PSGs may be directly down-regulating the production of TH1 cytokine transcripts, however it is more likely that the absence of TH1 cytokine production would be indirectly mediated via the effects of TH2 cytokines.

Currently the receptor(s) for PSGs is unknown. Identification of the receptor for PSG17 may assist in elucidating the signaling mechanisms that lead to the induction of TH2 cytokines. Based on the ability of PSGs to induce similar biological effects, human and murine PSGs may utilize the same receptor(s). Our data show there are an average of 1770 PSG17 binding sites per RAW cell with an approximate K_D of 2.2×10^{-11} M.

Although the number of receptors for PSG17 is low, the receptor-ligand interaction is of high affinity. Binding experiments with AP-PSG18N indicated a lower binding affinity for this protein, and about half the number of receptors per cell compared to PSG17 thus it is unlikely that they utilize the same receptors (data not shown). The difference in protein concentration required for cytokine induction by PSG17 and PSG18 also suggests different affinities for putative receptors. The 32% difference in amino acid sequence within the N-domains of PSG17 and PSG18 may result in recognition of unique binding partners for each of these PSGs.

A peptide derived from the N terminus of human PSG11, containing the RGD integrin-binding motif, bind to monocytes, but not to B or T cells (12). It was therefore proposed that the biological functions of PSGs involved binding of this motif in a manner similar to other RGD containing integrin binding partners (42). However, human PSG1, which lacks this motif (but has similar KGD sequence), induced IL-10, IL-6 and TGFβ_1 in human monocytes (13). Murine PSG17 and PSG18 contain an RGD-like RGE motif at the conserved site within their N-domains. Both of these proteins also induced cytokine secretion in macrophages indicating that the conserved RGD motif is not essential for biological function. However PSG binding to its putative receptor(s) may yet turn out to be dependent on the presence of this motif or similarly charged motifs such as RGE and KGD.

This is the first report to characterize the binding affinity for a murine PSG protein. Binding reached a plateau when RAW cells were treated with 86 nM AP-PSG17. We attempted to compare the binding affinity of PSG17 for RAW cells and peritoneal macrophages. Unfortunately, the binding curves obtained using peritoneal

macrophages were highly variable. All mice used in these experiments were BALB/c females between six and eight weeks old. Differences in immune responses during various stages of estrous have been reported for BALB/c mice (43). These mice reach sexual maturity between 40 and 60 days and the estrous cycle of these mice occurs every 4-5 days for approximately 10 hours. The variation in estrous cycle or sexual maturity may explain the irreproducibility of the binding data.

While the total concentration of human PSGs in maternal serum at term reaches 200-400 μg/ml, the physiological concentration of individual human PSGs is unknown. The homogeneity of this protein family prevents immunological identification of individual family members, because antibodies developed to human PSGs cross-react with multiple family members. There is no information available on the concentration of circulating murine PSGs because currently there are no immunological reagents available to identify murine PSGs. We are in the process of developing antibodies to recombinant murine PSG proteins, which will contribute to the study of these immuno-regulators.

In conclusion, our results indicate that murine PSG17 induces similar biological effects in macrophages to murine PSG18 and three human PSG family members. PSG functionality in macrophages, chromosomal location, structural similarities, and a common site of expression indicate that these genes have been highly conserved during evolution. Their high levels of expression in human pregnancy and decreased production in fetal pathologies suggest a critical role for PSGs in pregnancy. PSG antibody-induced abortion in animal models also contributes to this hypothesis. Induction of TH2 cytokines in macrophages by placentally produced PSGs may reduce inflammatory

immune responses and augment the generation of an immune environment compatible with successful pregnancy.

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III. Paper #2

Murine CD9 is the Cellular Receptor for Pregnancy Specific Glycoprotein 17

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Abstract

Pregnancy specific glycoproteins (PSGs) are a family of highly similar secreted proteins produced by the placenta. During gestation, they become the most prevalent pregnancy-related protein in maternal serum. Low concentrations of PSGs are associated with spontaneous abortion. It has been suggested that blood borne PSGs may act as immune mediators protecting the fetus from maternal immune responses. *In vitro* experiments performed in our lab, revealed that treatment of monocytes with human PSGs increased secretion of IL-10, IL-6, and TGFβ_1. This data supports such a hypothesis, providing an effective mechanism through which immune protection could occur. PSG homologues have been identified in several species including primates and rodents. Recently, we demonstrated that one murine PSG family member, PSG17, bound to RAW 264.7 cells, a murine macrophage cell line, with high affinity and induced secretion of anti-inflammatory cytokines in these cells. Based on these findings, we proposed that these cells express the PSG17 receptor. For the purpose of cloning the receptor, we screened a RAW cell cDNA expression library by panning. The PSG17 receptor was identified as the tetraspanin, CD9. We confirmed binding of PSG17 to CD9 by ELISA, flow cytometry, alkaline phosphatase binding assays, and in situ rosetting. Treatment with anti-CD9 antibodies inhibited binding of PSG17 to CD9 transfected cells. Two other murine PSGs, PSG18 and PSG19 were also tested for binding to CD9. They did not bind to cells transfected with this tetraspanin, suggesting that PSG17–CD9

binding is a specific interaction. We have identified the first receptor for a murine PSG as well as the first natural ligand for a member of the tetraspanin superfamily. With this discovery, we can identify the signaling mechanisms used by PSG17 to induce cytokine secretion in macrophages.

Introduction

Pregnancy specific glycoproteins (PSGs) are a family of highly similar, placentally secreted proteins, originally isolated from the circulation of pregnant women (1). PSGs are members of the Carcinoembryonic Antigen (CEA) family, which belongs to the Immunoglobulin superfamily. In humans, PSGs are secreted into the bloodstream and their concentration increases exponentially until term (2). Low levels of PSGs are associated with certain pathological conditions including spontaneous abortion, intrauterine growth retardation, and pre-eclampsia (3). Injections of anti-PSG antibodies induced spontaneous abortion in primates, indicating that PSGs are essential for successful pregnancy (4). Recently, we demonstrated that human PSGs induced IL-10, IL-6 and TGFβ_1 expression in human elutriated monocytes (5). It has been suggested that PSGs, through induction of these TH2 cytokines, may have a role in protecting the fetus from attack by the maternal immune system.

Despite their isolation from maternal serum about thirty years ago, the receptor(s) for these proteins has not yet been identified. Studies by Rutherford and colleagues reported a peptide derived from the N-terminal domain of human PSG11 binds to human monocytes and cells of the promonocyte lineage, but not to T or B cells (6). PSGs have also been identified in non-primates with hemochorial placentation including rats and mice. Screening of a YAC library revealed that there are 14 murine PSG genes (*PSG14-*

29) (7). These genes were mapped to chromosome 7 (8) and the cDNAs that have been characterized showed exclusive expression by the placenta (9).

We recently reported that murine PSG17 and PSG18 mimic the biological effects of human PSGs, by inducing TH2 cytokines in murine macrophages and RAW264.7, murine macrophage cell line (10, 11). We also showed that PSG17 binds to RAW cells with high affinity (11). Based on these findings, we proposed that these cells express a receptor for PSGs. For the purpose of cloning the putative PSG17 receptor, we screened a RAW cell cDNA expression library by panning. Positive clones were sequenced, and database queries revealed that the cloned binding partner for PSG17 is the tetraspanin CD9. We confirmed the specific binding of PSG17 to CD9 using four different techniques and in two different cell lines. PSG18 and PSG19 were also tested for binding to CD9. They did not bind to CD9 suggesting that the binding is a specific interaction between PSG17 and CD9.

CD9 is a member of the tetraspanin superfamily. This molecule is expressed on a wide variety of hematopoietic and non-hematopoietic cells. CD9 associates with other tetraspanin family members and integrins (12). To date, there are no known ligands for any of the more than thirty members of the murine and human tetraspanin superfamily (13, 14) (see also: www.ksu.edu/tetraspan/thepage.htm). Treatments with antibodies to different tetraspanins have implicated these cell surface proteins in cell migration, proliferation, activation and adhesion (reviewed by (13)). With such potentially diverse roles, they have been described as "molecular facilitators." This is the first report to identify the binding partner for a PSG family member, as well as a biological ligand for a member of the tetraspanin superfamily.

Materials and Methods

Reagents

Recombinant PSG17N-Myc-His, PSG18N-Myc-His, PSG19N-Myc-His and PSG6N-Myc-His were generated using a baculovirus expression system and purified from insect cell supernatant as previously described (5, 10, 11). Cell transfections were performed with Lipofectamine 2000 (Life Technologies, Rockville, MD). PSG17N-Myc-His coated plates were prepared as described (15). Briefly, under sterile conditions, bacteriological culture dishes (Falcon) were layered with 10 μg/ml goat anti- mouse IgG, extra serum absorbed (XSA) (KPL, Gaithersburg, MD) in phosphate coating solution (KPL). The plates were rinsed with PBS and blocked with bovine serum albumin (BSA) buffer (KPL). An anti-myc monoclonal antibody [1 μg/ml] (Invitrogen, Carlsbad, CA) was added to each dish followed by washes. The recombinant murine PSG17N-Myc-His proteins were subsequently added to the dishes.

Cell Culture

RAW 264.7 (American Type Culture Collection, Manassas, VA) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, 5mM sodium pyruvate (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (PSA) (Quality Biological, Gaithersburg, MD), and 10% Fetal Bovine Serum (FBS). HEK 293T (PEAK^{Rapid}) cells (Edge BioSystems, Gaithersburg, MD) were cultured in DMEM, 10% FBS, 50 μg/ml gentamicin, 250 μg/ml G418 (CalBiochem, La Jolla, CA), and PSA. EBNA 293 cells (Invitrogen) were grown in DMEM, 10% FBS, PSA and 250 μg/ml G418. BHK–21 cells (generously donated by

Dr. Gerardo Kaplan) were sustained in DMEM with 10% FBS and PSA. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Generation of a murine macrophage cDNA library

Total RNA was extracted from RAW 264.7 cells using TRIzol (Life Technologies) as per manufacturer's instructions. Purification of polyadenylated RNA and generation of the RAW 264.7 cell cDNA expression library in the PEAK10CV vector was done by Edge BioSystems (Gaithersburg, MD). The unamplified library yielded approximately 4.3 X 10^6 primary transformants with an average insert size of 1700 bp and an insert cut-off of 700 bp. For the first round of screening, 1.1 X 10^6 clones were plated on Luria-Bertani (LB) broth/agar plates with 100 µg/ml ampicillin (Sigma, St. Louis, MO). After 16 hours the plates were flooded with LB broth, the pooled bacteria were pelleted, and plasmid DNA was isolated by cesium chloride purification.

Recovery of cDNA clones by panning

Pooled purified plasmids were transfected into EBNA 293 cells (Invitrogen). Positive transfectants were selected using 0.5 µg/ml puromycin (Edge BioSystems). At 72 hours post selection, the cells were dislodged in PBS and 0.5 mM EDTA (16), and resuspended in binding buffer (PBS, 2% BSA). The detached cells were panned in PSG17N-Myc-His coated petri dishes at 1.0 - 1.5 X 10^7 cells per dish (16, 17). Non-adherent cells were removed by washing with at least six PBS/BSA washes until there were no floating cells visible under the microscope. Adherent cells were removed from the bottom of the dishes by pipetting and seeded into in poly-L-lysine coated wells.

Puromycin was increased to 1 μg/ml two weeks after the initial transfection. An additional two rounds of panning were performed before the episomal plasmids were isolated using the HIRT extraction procedure. The plasmids were transfected into high efficiency ElectroMAX^{TM} DH10B cells (Life Technologies) by electroporation. Twelve random single colonies were grown and the plasmids purified using Qiagen Midi-Prep columns. Individual plasmids were transfected into 293T cells and the transfected cells were screened for binding of PSG17N-Myc-His by ELISA (see below). Inserts from the plasmids that conferred PSG17N binding were sequenced with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits (PE Biosystems, Foster City, CA).

Detection of PSG17 Binding to transfected HEK 293T cells by ELISA

For the identification of the PSG17 putative receptor, HEK 293T cells were seeded in poly –L lysine coated 96 well plates at 5 X 10^4 cells per well and transiently transfected with plasmid DNA obtained as described above. At 48 hours post-transfection, the cells were washed with binding buffer containing 0.01% sodium azide and PSG17N-Myc-His (10 μg/ml) (or no ligand) was added to each well. After 1 hour of incubation at room temperature, the ligand was aspirated and the cells were washed 5 times with binding buffer without sodium azide. To detect binding of PSG17N-Myc-His, anti-myc-horseradish peroxidase (HRP) conjugated antibody (Ab) was added to the cells for 1 hour at room temperature at a concentration of 1 μg/ml (in binding buffer). Binding of the antibody to the ligand was detected after the addition of tetramethylbenzidine (TMB)-peroxidase substrate (KPL) followed by 2N H_2SO_4 (stop solution). The color change was quantitated at 405 nm on a microplate spectrophotometer.

Competition experiments were carried out in CD9-pEF6/V5-His (Invitrogen) transfected 293T cells by adding increasing concentrations of anti-murine CD9 Ab (BD Pharmigen, San Diego, CA) or an isotype matched control (rat IgG_{2a}) for 1 hour at room temperature prior to treatment with 5 μg/ml PSG17N. Absorbance was normalized to background binding of PSG17N in the presence of anti-CD9 antibody to empty plasmid transfected 293T cells. To examine whether other murine PSGs bound to CD9, ELISAs were also performed using PSG18N-Myc-His and PSG19N-Myc-His at 10 μg/ml in place of PSG17N-Myc-His.

In Situ Rosetting Assay

In situ rosetting assays modified from Kaplan *et al.* were performed to confirm binding of PSG17 to CD9 (18). Briefly, 293T cells transiently transfected with empty vector or CD9-pEF6/V5-His were seeded at low density into poly L-lysine coated 60 mm dishes (Becton Dickinson, Sparks, MD). Attached cells were washed in binding buffer (PBS- 2% BSA). PSG17N-Myc His (90 picomoles) or binding buffer alone was added to the dishes for 1 hour at room temperature. The buffer was aspirated from each dish and the cells were washed four times with PBS to remove any unbound ligand prior to the addition of 1 μg/ml anti-myc Ab in binding buffer for 1 hour at room temperature. As a control, the anti-myc Ab was omitted from some plates. Dishes were washed again with PBS prior to the addition of 15 μg of rabbit anti-mouse immunoglobulin coated beads (BioRad, Richmond, CA). Unbound beads were removed by washing extensively with PBS and receptor positive cells were viewed by microscopy.

Flow cytometry:

HEK 293T or BHK-21 cells were transfected with murine CD9-pEF/V5-His or empty vector. Transfected cells were divided into polystyrene tubes at 5 X 10^5 cells/tube. Cells were washed twice with wash buffer (PBS; 3% FBS; 0.01% sodium azide), prior to the addition of 10 μg PSG17N-Myc-His for 30 minutes at room temperature. After two washes, cells were incubated on ice sequentially with the following solutions for 30 minutes each: (see Table 1 & 2) 0.5 μg anti-myc Ab (Invitrogen), 0.5 μg goat anti-mouse IgG_{2a} kappa chain-biotin labeled Ab (BD Pharmigen) and 0.5 μg streptavidin-conjugated FITC (BD Pharmigen) with two washes between each incubation. PSG17N binding to HEK 293T and BHK 21 cells was analyzed by flow cytometry using an EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL) and the percent binding was determined with the System II Software program (Beckman Coulter). Overlays were produced with the WinList program (Verity Software House, Topsham, ME).

Alkaline phosphatase binding assays:

PSG alkaline phosphastase (AP) fusion proteins were generated by cloning into the AP-Tag4 vector generously supplied by Dr. John Flanagan (Harvard University). CD9 binding assays with heat stable AP-PSG fusion proteins were performed as described by Flanagan and Cheng (19). 293T cells transiently transfected with murine CD9 or empty vector were cultured in poly-L-lysine coated six well plates. Increasing concentrations (43, 86 and 129 nM) of AP-PSG17 or the AP control protein were added to each well in triplicate for 90 minutes at room temperature. The cells were thoroughly washed, and the concentration of bound protein was measured from cleared, heat-

inactivated cell lysates by a colorimetric reaction as previously described (11). Binding of AP-PSG18N to CD9 transfected cells was also investigated at two concentrations, 8.6 nM and 86 nM.

Results

Identification of CD9 as the cellular receptor for PSG17

Based on the ability of PSG17 to induce cytokines in the mouse macrophage cell line, RAW264.7, and the high binding affinity of PSG17 for a ligand on the surface of these cells, we proposed that these cells expressed the receptor for PSG17. To isolate this receptor, a RAW cell cDNA library was generated (Edge BioSystems) in the PEAK10CV vector. Plasmid DNA from ~1.1 X 10^6 unamplified clones was transfected into 293 EBNA cells and transfected cells were selected by addition of puromycin, prior to panning on PSG17N-coated dishes. After three rounds of panning, plasmid DNA was isolated by HIRT extraction and electroporated into ElectroMAX DH10B *E. coli*. Plasmid DNA isolated from individual bacterial colonies was re-transfected into HEK 293T cells and their ability to confer PSG17 binding was determined by ELISA. All positive clones were sequenced. Database queries identified a perfect match between these cDNAs and the tetraspanin family member, murine CD9.

To confirm binding of PSG17 to CD9, CD9-pEF6/V5-His transfected 293T cell were treated with PSG17N-Myc-His and analyzed by ELISA. PSG17N-Myc-His was shown to bind CD9 transfected cells in a dose responsive manner with a binding plateau at 10 μg/ml (Figure 1). In the cells transfected with empty plasmid, there was only a slight increase in background binding with increasing PSG protein concentration. Non-

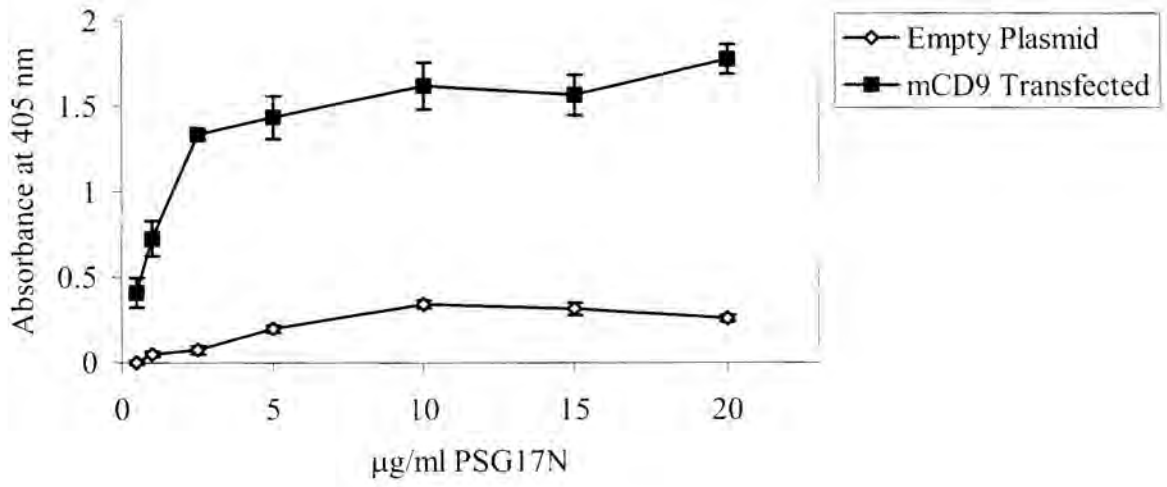

Figure 1. Binding of PSG17N to murine CD9 transfected 293T cells. HEK 293T cells were transfected with CD9-pEF6/V5-His or empty plasmid. Forty-eight hours after transfection, increasing concentrations of PSG17N-Myc-His were added for 1 hour at room temperature. Binding of PSG17 was detected after treatment with HRP conjugated anti-myc Ab followed by addition of TMB/peroxidase substrate. The data is expressed as mean absorbance ± S.E. Each data point represents five identical wells from three independent experiments

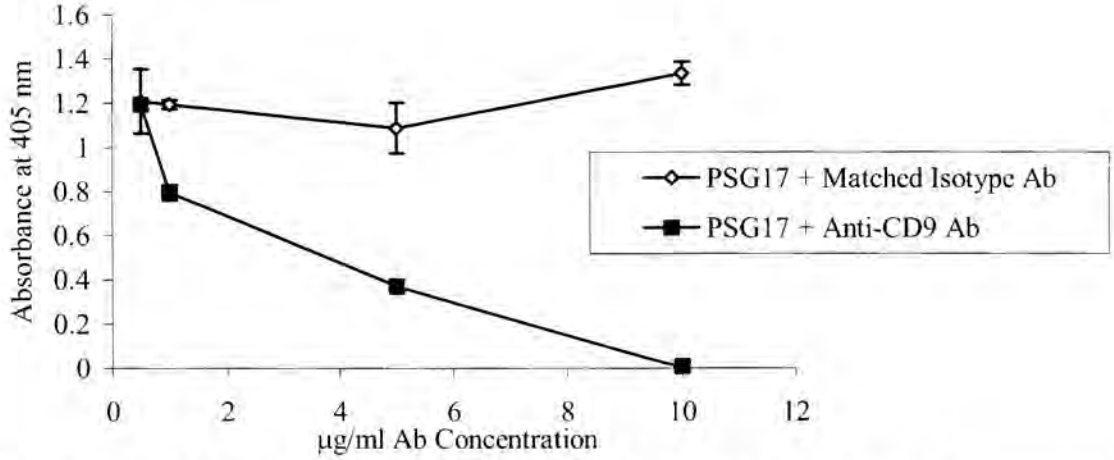

Figure 2. Anti-CD9 antibodies inhibit PSG17 binding. HEK 293T cells transfected with murine CD9-pEF/V5-His were treated with increasing concentrations of an anti-CD9 Ab or an isotype matched control Ab. PSG17N-Myc-His was added at a fixed concentration of 5 µg/ml to each well. Unbound PSG17 was washed from the well and HRP conjugated anti-myc Ab was added. Each data point represents four wells and is expressed as mean absorbance ± S.E.

specific binding of the anti-myc Ab to CD9 and control transfected cells was similar to binding of 5 μg/ml PSG17N to control cells (data not shown). This indicates that the binding reported in the control cells reflects non-specific binding of the secondary antibody. Rosetting experiments further confirmed binding of PSG17N-Myc-His to CD9. CD9-pEF6/V5-His transfected cells treated with PSG17N-Myc-His and anti-myc Ab and rabbit anti-mouse Ig immunobeads showed a rosetting pattern. This pattern was not observed in the control dishes, lacking either PSG17N treatment, anti-myc Ab or expression of CD9 (data not shown).

To demonstrate specific binding between PSG17N and murine CD9, competition ELISAs were performed in HEK 293T cells transfected with empty vector or CD9-pEF6/V5-His (Figure 2). Forty-eight hours after transfection, anti-CD9 antibody or a matched isotype control were added followed by PSG17N-Myc-His. The addition of 10 μg/ml anti-CD9 Ab reduced PSG17N binding to background levels. PSG17 binding to CD9 was also analyzed by flow cytometry (Table 1; Figure 3). HEK 293T cells were transfected with CD9 -pEF6/V5-His or empty vector. Treatment with PSG17N or other control proteins and a series of antibodies followed (the combinations of antibodies are displayed in Table 1.) To determine extent of expression of CD9 in the transfected cells, the cells were treated with biotin labeled anti-CD9 Ab or a matched isotype. Approximately 70% of the cells stained positive for CD9 (lane 16 figure 3A). The percentage of cells that stained positive for PSG17 binding to CD9-transfected cells ranged from 66-87 % while there was less than 1% binding to the empty vector transfected cells (Figure 3A and B). Screening of the library by panning and binding experiments had been performed on cells of the same lineage. To examine whether

PSG17 binds to CD9 on cells of a different species, we transfected BHK-21 cells with CD9-pEF6/V5-His. The results show significant binding of the PSG17N to CD9 transfected BHK-21 cells (~97%) compared to controls (<5%) suggesting that CD9 expression may be sufficient for PSG17N binding in any cell type (Table 2)(Figure 4).

Table 1. Treatments used in Flow Cytometric Analysis of PSG binding to CD9 in HEK 293T

Transfected Cell Type	1st Incubation	2nd Incubation	3rd Incubation	Detection
1. 293T (empty vector)	X[1]	X	X	X
2. 293T (empty vector)	X	X	X	Strep-FITC[2]
3. 293T (CD9)	X	X	X	Strep-FITC
4. 293T (empty vector)	PSG17N[3]	Anti-Myc	G∝M IgG-Bio[4]	Strep-FITC
5. 293T (CD9)	PSG17N	Anti-Myc	G∝M IgG-Bio	Strep-FITC
6. 293T (empty vector)	X	"	"	"
7. 293T (CD9)	X	"	"	"
8. 293T (empty vector)	PSG6N	"	"	"
9. 293T (CD9)	PSG6N	"	"	"
10. 293T (empty vector)	PSG18N	"	"	"
11. 293T (CD9)	PSG18N	"	"	"
12. 293T (empty vector)	PSG19N	"	"	"
13. 293T (CD9)	PSG19N	"	"	"
14. 293T (empty vector)	Anti-CD9-Biotin	Y[5]	Y	"
15. 293T (empty vector)	Rat IgG2$_{ak}$Biotin	Y	Y	"
16. 293T (CD9)	Anti-CD9-Biotin	Y	Y	"
17. 293T (CD9)	Rat IgG-k Biotin	Y	Y	"

[1] X: Wash buffer
[2] Strep-FITC: Streptavidin-FITC (0.5 µg)
[3] All PSG proteins are Myc-His labeled: 10 µg were added to each tube
[4] G∝M IgG-Bio: Goat Anti-Mouse IgG$_{2ak}$-Biotin Labeled (0.5 µg)
[5] Y: No incubation step

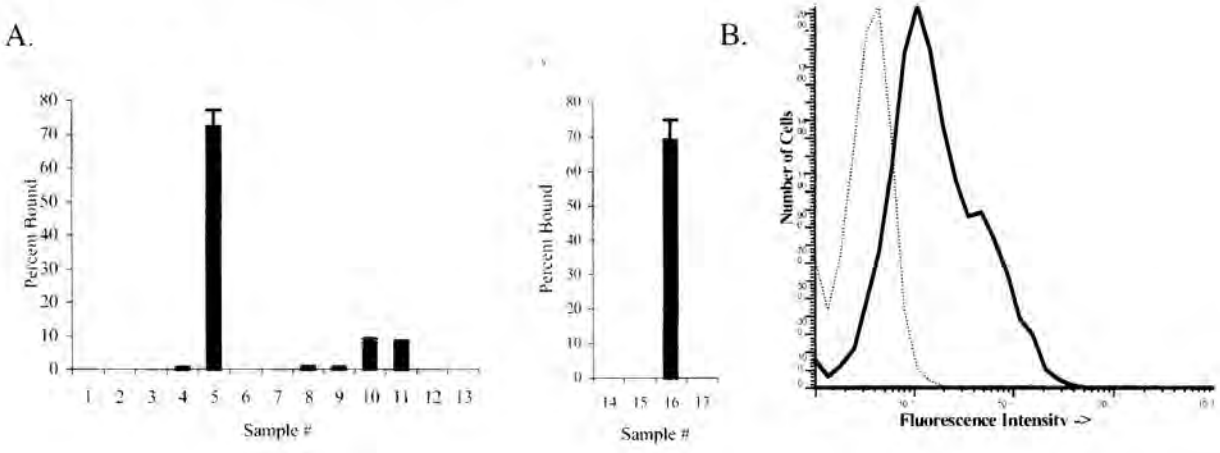

Figure 3. FACS analysis of PSG17N-Myc-His binding to CD9 transfected 293T cells. (A) 293T cells transfected with empty plasmid or murine CD9-pEF6/V5-His were treated with PSG17N-Myc-His, anti-myc antibody, biotin conjugated goat anti-mouse IgG$_{2a}$κ and FITC conjugated-streptavidin. The percent binding was determined by flow cytometry. Each sample without a bar represents an average binding of <1%. See Table 1 for treatment protocol. (B) Alignment of flow cytometry reports for binding of PSG17N to empty plasmid transfected 293T cells (#4)(dotted line) and binding of PSG17N to the CD9 transfected cells (#5)(solid line).

Table 2. Treatment protocol for PSG binding to CD9 in BHK-21 cells by flow cytometry

Transfected Cell Type	1st Incubation	2nd Incubation	3rd Incubation	Detection
1. BHK21(empty vector)	X[1]	X	X	X
2. BHK21(empty vector)	X	X	X	Strep-FITC[2]
3. BHK21(CD9)	X	X	X	Strep-FITC
4. BHK21(empty vector)	PSG17N[3]	Anti-Myc	G∝M IgG-Bio[4]	Strep-FITC
5. BHK21(CD9)	PSG17N	Anti-Myc	G∝M IgG-Bio	Strep-FITC
6. BHK21 (CD9)	PSG18N	"	"	"
7. BHK21(empty vector)	Anti-CD9-Biotin	Y[5]	Y	"
8. BHK21(empty vector)	Rat $IgG2_{ak}$Biotin	Y	Y	"
9. BHK21(CD9)	Anti-CD9-Biotin	Y	Y	"
10. BHK21(CD9)	Rat $IgG2_{ak}$Biotin	Y	Y	"

[1] X: Wash buffer
[2] Strep-FITC: Streptavidin-FITC (0.5 μg)
[3] All PSG proteins are Myc-His labeled; 10 μg were added to each tube
[4] G∝M IgG-Bio: Goat Anti-Mouse IgG_{2a}-Biotin Labeled (0.5 μg)
[5] Y: No second and third incubation steps

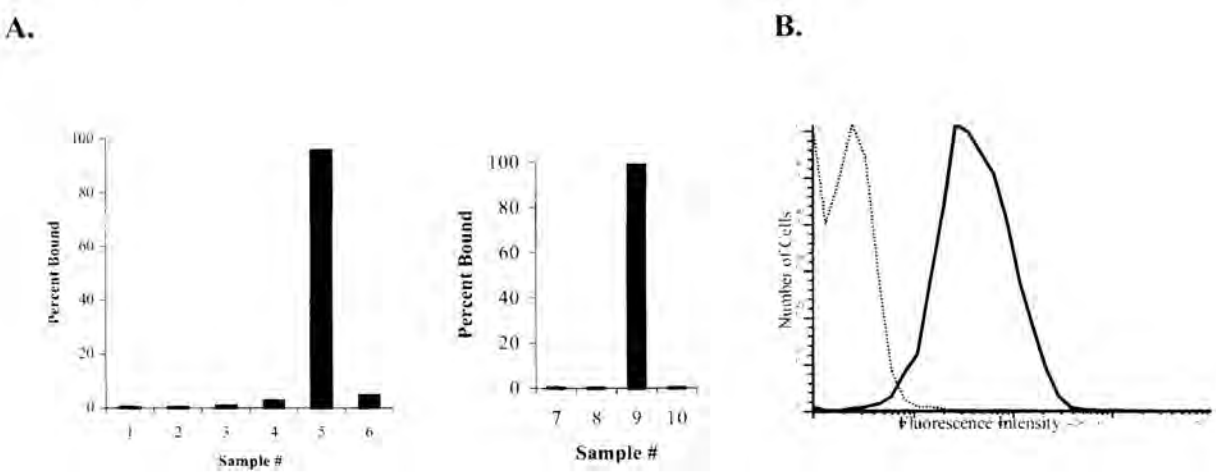

Figure 4. FACS analysis of PSG17 binding to BHK-21 cells expressing murine CD9. (A.) BHK-21 cells transfected with empty plasmid or murine CD9-pEF6/V5-His were treated with PSG17N-Myc-His, anti-myc antibody, biotin conjugated goat anti-mouse $IgG_{2a}\kappa$ and FITC conjugated-streptavidin (Table 2). The percent binding was determined by flow cytometry. Sample #9 demonstrates expression of CD9. (B.) The dotted line represents empty plasmid transfected 293T cell treated with PSG17N (#4) and the solid line represents binding of PSG17N to the CD9 transfected cells (#5).

Binding of Alkaline phosphatase-PSG17 (AP-PSG17) to CD9

The previously presented binding experiments utilized a truncated N-domain of PSG17. We examined binding of the full length PSG17 to murine CD9 using a recombinant AP-PSG17 fusion protein. HEK 293T cells were transfected in 6 well poly-L-lysine coated dishes and treated with increasing concentrations of AP-PSG17 or an AP control protein (Figure 5). Full length PSG17 binding to CD9 transfected cells was concentration dependent while binding of the AP-control protein remained at baseline levels.

Murine PSG18 and Murine PSG19 do not bind to CD9

We have previously shown that PSG18 induces cytokine secretion in RAW cells (10) and preliminary data from our lab showed that PSG19 has the same effect suggesting that receptors for PSG18 and PSG19 are present on these cells. To determine if these murine PSGs utilize the same binding site as PSG17, we tested binding of these proteins to CD9 transfected 293T cells. Binding of PSG18 and PSG19 to CD9 was investigated using AP-fusion proteins, flow cytometry, or ELISA. In the AP-binding assays, CD9-pEF6/V5-His transfected cells were treated with two different concentrations of AP-PSG17, AP-PSG18N or the AP-control protein. Binding of PSG18N was equivalent to that of the AP control protein (Figure 6). PSG18N and PSG19N also did not show significant binding to CD9 transfected cells by flow cytometry (Figure 3A and 4A) and by ELISA (data not shown). Our results indicate that these two murine PSGs do not bind to the tetraspanin CD9.

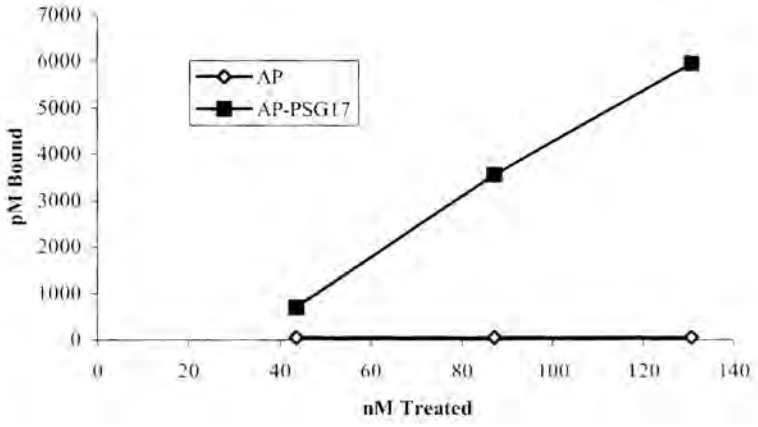

Figure 5. Full length PSG17 binds to CD9 transfected cells. 293T cells transfected with CD9-pEF6/V5-His were treated with increasing concentrations of AP-PSG17 or the AP-control protein. Each experiment was done in triplicate and the data points represent the mean concentration bound. The standard error was less than 25 pM for all samples.

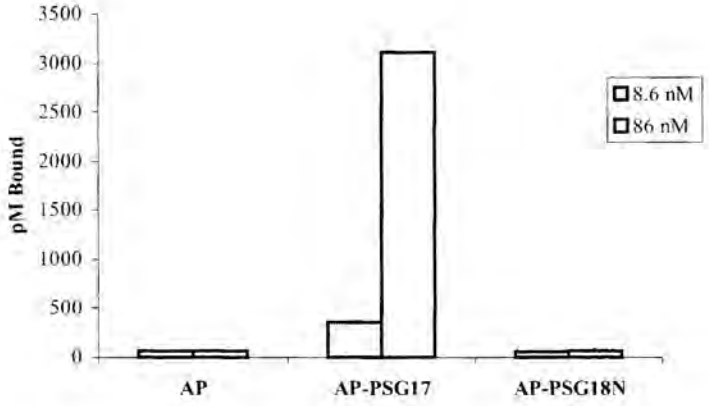

Figure 6. PSG18N and PSG19N do not bind to CD9. 293T cells were transiently transfected with murine CD9-pEF6/V5-His. AP-fusion proteins were added for 90 minutes at room temperature. The concentration of bound protein was determined as previously described. Each bar represents the mean of at least 3 data points. Standard error was less than 15 pM for all samples.

Discussion

In this report, we identified the first binding partner for a murine PSG family member as well as the first natural ligand for a member of the tetraspanin superfamily. Using various methods, we showed that murine PSG17 binds to the tetraspanin family member CD9. The binding between PSG17 and CD9 is specific, as it can be inhibited with increasing concentrations of anti-CD9 antibody. Two other murine PSGs, PSG18 and PSG19 do not associate with this receptor. This is very intriguing because murine PSG18 and PSG19 have conserved biological functions with PSG17, however these effects may not be triggered through identical receptors.

Sequence analysis of CD9 suggests that like other tetraspanins, there are four hydrophobic transmembrane domains. These four domains create two extracellular loops, a small and long one, with two short intracytoplasmic tails at the amino and carboxyl termini (20). In the plasma membrane, CD9 associates with other tetraspanin family members including CD53, CD63, CD81, CD82, and CD151 (21, 22). CD9 has also been immunoprecipitated with several β–integrins (23, 24). Other cell surface molecules present in tetraspanin complexes include HLA-DR (22) and MHC Class II glycoproteins (25). PSG17 could signal induction of cytokines by means of variety of interactions with any of these molecules or others (13). To date, the complexes associated with CD9 in murine macrophages have not been determined.

Human and murine CD9 display 89% homology at the amino acid level (26), which together with the conserved function of human and murine PSGs suggests that at least one of the human PSGs could also utilize this binding partner. We have tested recombinant human PSG1, PSG6 and PSG11 proteins for binding to 293T cells over-

expressing human CD9. None of these three glycoproteins associated with this tetraspanin. Co-immunoprecipitation experiments with a peptide derived from the N-domain of human PSG11 identified a 46-kDa protein from THP-1 cell membranes as a putative binding motif on these cells for PSG11 (6). Interestingly, some human tetraspanins have similar apparent molecular weights as the binding molecule identified in the human monocytic cell line. Presently, we are testing whether any of these three human PSGs bind to other tetraspanin family members, however, it is possible that human PSGs have evolved to utilize a different receptor entirely.

Two groups have independently generated CD9 knockout mice and discovered that these mice have severe fertility defects (27). CD9 expression on oocytes is utilized for sperm-egg fusion resulting in reduced fertility for CD9-/- females. We expect that treatment of peritoneal macrophages from CD9 knockout mice with PSG17 will not result in the up-regulation of IL-10, IL-6 and TGFβ_1, however we may also find that other receptor(s) may also bind PSG17 and result in cytokine induction. CD9 -/- mice can produce some viable fetuses, indicating that CD9 is important for fertilization but is not essential for pregnancy success. This observation is consistent with the fact that although murine PSGs seem essential during pregnancy, they have evolved to use different receptors and no single PSG may be critical. Also we anticipate that PSG-mediated cytokine secretion would be more crucial in matings of mice with different genetic backgrounds.

Human CD9 is expressed on the surface of extravillous trophoblasts (23). With its association to the integrin α_5, human CD9 is suspected to play an important role in regulation of trophoblast invasion in humans. The possible role of CD9 in invasion of the

murine placenta remains to be investigated. The association of PSG17 and CD9 has linked the goals of better defining PSG immuno-regulatory control of the innate immune system during pregnancy and determining the signaling events following binding to a murine tetraspanin.

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IV. Discussion

PSG Induction of Cytokines in Pregnancy

IL-10 and Macrophages

Macrophages are required for implantation in mice [191] and macrophages are prevalent in the uterus during pregnancy [141, 192]. We showed that the N-domain of PSG17 induced secretion of IL-10 in BALB/c thioglycollate-induced peritoneal macrophages and in RAW 264.7 cells, a murine macrophage cell line. A minimum concentration of 10 μg/ml was necessary to up-regulate IL-10 production, and the cytokine response was dose-dependent in RAW 264.7 cells. Uterine macrophages have been characterized as "immunosuppressive" during pregnancy [193]. Macrophage-secreted IL-10 can down-regulate inflammatory cytokines produced in activated human and murine mononuclear cells such as IFN-γ, TNFα, granulocyte macrophage colony stimulating factor (GM-CSF), IL-1 and IL-12 [194-196]. IL-10 also inhibits the expression of IL-8 and G-CSF at both the mRNA and protein levels [197]. In addition, IL-10 regulates the expression level of cell surface molecules required for antigen presentation on certain classes of human monocytes [198]. Even in the presence of IL-4 or IFN-γ, IL-10 decreases the expression of MHC class II molecules on LPS-activated monocytes inhibiting antigen presentation and thus inhibiting T cell activation [199]. In the mouse model of RSA, inflammatory cytokines such as IFNγ and TNFα are associated with spontaneous abortion [107]. Exogenous IL-10 can suppress these cytokines and prevent abortion in these mice [102]. The ability of PSGs to induce IL-10 synthesis in macrophages suggests that PSGs may play a role in down-regulating inflammatory

immune responses to produce the TH2-like immune environment that is thought to prevent fetal rejection.

IL-10 Expression in Trophoblasts

Placental trophoblast cells constitutively produce IL-10 *in vitro* and *in vivo* [98, 99, 132]. Production of IL-10 by these cells may also contribute to the TH2 cytokine profile observed during pregnancy [200]. Trophoblast cells treated with exogenous IL-10 showed a decrease in matrix metalloproteinase (MMP)-9 secretion and invasion *in vitro* [201]. MMPs are a family of extracellular matrix degrading enzymes with functional roles in tissue remodeling. These experiments suggested that trophoblastic IL-10 may regulate trophoblast invasion of the uterine wall. Conversely, IL-10 may have a dual role in regulating trophoblast invasion, because decreased production of IL-10 is associated with preeclampsia, which is characterized by shallow invasion of the placenta into the uterine wall [202, 203]. Reduced serum concentrations of PSGs are also associated with preeclampsia in women [7]. Therefore, PSGs may not only influence the immune system response during pregnancy, but may help control placental invasion through their ability to regulate IL-10 production. As described later, PSGs may also use autocrine pathways to influence the ability of trophoblasts to secrete IL-10.

Interleukin 6 and Pregnancy

IL-6 is a multifunctional cytokine with diverse biological activities including regulation of hematopoiesis, and proliferation and function of B and T cells, macrophages and hepatocytes. Besides lymphocytes, macrophages/monocytes, microglia, astrocytes

and mast cells, IL-6 is produced by many non-immune-related cells and organs, including trophoblasts and endothelial cells [204]. There is a significant increase in IL-6 expression by uterine epithelial cells and macrophages at the beginning of pregnancy [205, 206]. In mice, IL-6 bioactivity is greatest on days 5 and 6 of pregnancy [90]. *In vivo* experiments with IL-6 knockout mice demonstrated that this cytokine is important for immune response regulation. In the absence of IL-6, an over zealous secretion of inflammatory cytokines occurs after treatment with LPS or bacterial infection [207, 208]. PSG induction of IL-6 in macrophages could moderate inflammatory immune responses, which are believed to be harmful to the fetus.

The Importance of TGF-β1 during Pregnancy

The importance of TGF-β in the induction and maintenance of normal pregnancy is suggested by TGF-β-knockout mice. These animals have severe, spontaneous, multi-organ, autoimmune disease associated with autoantibody production, which proves fatal in early life [209, 210]. TGF-β has multiple immunosuppressive effects at the cellular level, inhibiting B cells, CD4+ and $CD8^+$ T cells, macrophages and natural killer cells [211]. We showed that treatment with only 1 μg/ml PSG17N induced production of $TGF\beta_1$ in RAW 264.7 cells. Albeit with different kinetics, PSG17 treatment of peritoneal macrophage also resulted in an up-regulation of $TGF\beta_1$. PSG induction of $TGF\beta_1$ could further contribute to the immunoregulatory effects induced by IL-10. TGFβ can also downregulate MHC class II expression on macrophages thereby suppressing the ability of APCs to activate T cells.

Prostaglandin E_2 and Pregnancy

Prostaglandins are eicosanoids or lipid mediators. Prostaglandins are potent paracrine hormones that are essential for a variety of functions affecting both fetal and maternal tissues during pregnancy [212, 213]. PGE_2 can upregulate the levels of both IL-10 and IL-6 produced by macrophages [214], which made it an ideal potential mediator for induction of both of these cytokines. Furthermore, PGE_2 inhibits production of TH1 cytokines and has been suggested to play a role in supporting TH2 immune responses [215-217]. The production of IL-10 has a negative feedback effect on PGE_2 secretion by macrophages, which may explain why the levels of PGE_2 in RAW cell supernatants were not elevated at six and twenty-four hours post-treatment. The ability of PSG17 to induce this eicosanoid in macrophages provides an additional mechanism for suppression of immune responses that are potentially harmful to the fetus.

CD9- PSG17 Interaction

To isolate the putative PSG17 receptor, a RAW 264.7 cell cDNA expression library was constructed. Our binding experiments indicated that RAW cells expressed approximately 1770 PSG17 binding sites per cell with a K_D of 2.2×10^{-11} M. Although the receptor number was lower than in peritoneal macrophages, the initial binding data we obtained with RAW 264.7 cells was more reproducible than that obtained from primary macrophages. Therefore, due to both the reproducibility of binding and the ease of preparation of good quality mRNA needed for the library, we decided to use the cell line as a source of RNA.

To identify the ideal cell line to use for the transfection of the library, we looked at several different parameters. First and most importantly, the cell line had to be PSG17-receptor negative and had to have low background binding to PSG17 or the anti-myc antibody used to coat the plates. Second, the cells would have to be transfected with high efficiency and ideally would maintain the plasmid episomally for easier recovery. Other important features were the mortality rate after treatment with the selection reagent and the time required for selection. In other words, we were interested in being able to select in a fast and reliable way, only the cells that were transfected with library DNA.

We determined that human 293 cells did not bind PSG17 using an AP-binding assay (described in paper #1). The AP control protein and AP-PSG17 showed identical binding to these cells. Background binding was lower in 293 cells when compared to other putative receptor negative cells such as NIH 3T3 cells. To ensure the highest level of transfection efficiency, we tested a number of transfection reagents using a GFP-reporter plasmid (generously supplied by Dr. Silvio Gutkind). This plasmid used the same promoter, elongation factor 1-α, to drive expression of the GFP cDNA, as present in the vector the RAW cell library was in. The transfection efficiency in EBNA 293 cells was subjectively scored using a fluorescent microscope 24 and 48 hours post transfection. The highest percentage of transfected cells was obtained using Lipofectamine 2000 (Life Technologies), followed closely by GenePORTER (Gene Therapy Systems, San Diego, CA) with between 85-95% positives. Calcium phosphate transfection was by far the least effective method (40-50% positives). For the establishment of stably transfected cells, 293 EBNA cells were treated with 0.5 μg/ml puromycin, the antibiotic resistance gene present in the library plasmid. Puromycin was added 48 hours post-transfection and its

concentration was increased to 1 μg/ml one week post-transfection. As demonstrated using the GFP control, this regimen resulted in effective killing of the non-transfected cells by three days with no toxicity for the transfected ones.

Once the cell line was selected and the different parameters were worked out, we proceeded with screening of the library as described in paper #2. Sequencing of clones that conferred PSG17 binding on transfected 293T cells revealed that the receptor for PSG17 was the tetraspanin CD9. We confirmed the CD9-PSG17 binding by ELISA, flow cytometry, AP binding assays and *in situ* rosetting. We analyzed whether transfection of CD9 resulted in PSG17 binding in a PSG17- receptor negative cell from a different species, BHK-21 cells. In addition, we attempted to transfect NIH3T3 cells, but the transfection efficiency, for the GFP- control vector was significantly lower in these cells so they were not used.

In parallel with the library screening, we designed experiments aimed at the characterization of the size of the putative receptor(s). Although Mansfield and coworkers had identified a single molecule binding to human PSG11 in THP-1 cells, a human promonocytic cell line [51], we also wanted to verify that the murine PSG17 receptor was composed of a single polypeptide chain. For this purpose, RAW cell membranes were labeled with NHS-LC Biotin (Pierce). To isolate the receptor(s), the biotinylated membranes prep was passed through a PSG17N-Myc-His-nickel-NTA column (Qiagen). After several washes, the bound proteins were separated from the beads by boiling in 6X SDS loading buffer. The proteins were separated on a gradient SDS-PAGE gel, prior to Western blot analysis. The membrane proteins on the nitrocellulose membranes were treated with streptavidin-horseradish peroxidase (HRP)

and detected on film by chemiluminescence. To identify proteins which were non-specifically bound, the assay was repeated several times using Ni-NTA beads coated with PSG17N or psp-amis, a histidine-labeled control protein from the *Pseudomonas putrida* (supplied by Luva Grinberg, USUHS). Three different "putative" receptors were identified in this manner at 58, 42, and 27 kDa. (The other proteins may have non-specifically bound to the beads or were components of PSG receptor containing "membrane rafts" that sometimes form in the presence of mild detergents. Detergent strengths were varied with each successive column in attempts to prevent this phenomenon from occurring.) To confirm PSG17N binding to the "putative" receptor protein on the blot, PSG17N-Myc-His was added to the membrane, and anti-myc Ab was used to detect binding. Unfortunately, the anti-myc Ab would also detect contaminating PSG17N-Myc-His on the blot obtained from the affinity column upon boiling. Due to the similar size of CD9 and PSG17N-Myc-His, this approach would not have allowed us to distinguish between presence of the 24 kDa CD9 and the presence of the PSG17N-Myc-His in the gradient gel.

It will be important to determine whether CD9 is a component of the signal pathway leading to PSG17 mediated cytokine secretion in macrophages. To this end, we are currently breeding CD9+/- heterozygotes (generously provided by Dr. Claude Boucheix) to produce CD9 knockout mice. We expect that PSG17 treatment of peritoneal macrophages from CD9 -/- mice with PSG17 will not result in the up-regulation of IL-10, IL-6 and TGFβ_1. Treatment of CD9-/- macrophages with LPS will be used as a positive control for cytokine secretion. In the event that PSG17 induces

cytokine production in the absence of CD9 expression, further screening the cDNA library for additional receptors will be done.

Sites of Expression of CD9

The discovery of the PSG17-CD9 interaction has increased the importance of identifying sites of expression of this tetraspanin, particularly with regard to cells of the immune system. Oritani *et al* has already reported CD9 expression on a variety of hematopoietic cell types. Two-dimensional FACS analysis of peripheral lymphocytes from mouse spleen identified CD9 expression on B and T cells [177]. Murine CD9 was also found on peripheral blood platelets, neutrophils and mature myeloid cells [177]. CD9 was identified on eosinophils [170] and on acute non-lymphoid leukemias [175]. In addition, CD9 expression was confirmed in RAW 264.7 cells (data not shown) and in cultured dendritic cells (Dr. Jesus Colino, USUHS, *personal communication*).

CD9 and T cells

T lymphocytes are a key component of the immune response. CD9 is present on activated T cells. Therefore, to widen the investigation of PSG effects to include the adaptive immune system, future functional studies of PSG17 should also include T cells. Fujiwara and colleagues demonstrated proliferation of naïve murine T cells in the presence of anti-CD3 and anti-CD9 antibodies [218]. Within 72 hours, apoptosis of anti-CD3 and anti-CD9 Ab treated cells occurred while cells treated with anti-CD3 and anti-CD28 Ab remained viable [180]. The CD9- CD3- induced activation of T cells only transiently up-regulated IL-2 production (less than 24 hours), whereas anti-CD28

stimulated cells constitutively secreted IL-2. The anti-CD9/anti-CD3 Ab activated cells were rescued from apoptosis when exogenous IL-2 was added to the cultures [180]. Contrary to these results, when the human T cell line Jurkat is activated with anti-CD3 Ab and then treated with anti-CD9 Ab these cells secrete IL-2 and no apoptosis occurs [181]. The role of CD9 in T cell proliferation should be further investigated using its natural ligand.

PSG17 binding to CD9 on T cells may result in the modulation of cytokine induction. To determine the effects of PSG17 on T cells, mixed lymphocytes and/or purified T cells from CD9 knockout mice will be stimulated with anti-CD3 and/or anti-CD28 in the presence of PSG17, and cytokine secretion and cell proliferation will be examined. Preliminary data on mixed lymphocytes from BALB/c mice treated with PSG17N revealed an inhibition of proliferation of activated cells in the presence of PSG17. A reduction in proliferation was observed in cells treated with PSG17 and CD3 and/or CD28 implying that murine PSGs could repress activation of these immune cells. IL-10 suppresses expression of the co-stimulatory ligands for CD28, CD80 and CD86, thereby abrogating interactions that are essential for T cell activation [Ding, 1993 #530; [219]. IL-10 also induces alloantigenic-specific unresponsiveness in human CD8+ T cells activated in the presence of professional APC [220]. The ability of PSGs to induce macrophage IL-10 secretion may prevent CD8+ T cells from proliferation after antigen presentation. Studies using purified T cells will demonstrate whether the effects of PSG17 on proliferation are dependent on IL-10 secretion by APCs. Suppression of maternal T cell activation has been reported during pregnancy, however the mechanisms that inhibit proliferation to prevent fetal reactivity are speculative. We propose that

PSG17 is inducing IL-10 secretion in macrophages and potentially also in T cells through interaction with CD9. This increase in IL-10 production could be a contributing factor to regulation of T cell activation during pregnancy. IL-10 inhibits IL-2 and TNFα production in anti-CD3 activated T cells in the absence of APCs [198, 221]. The ability of PSG17 to induce IL-10 secretion in activated T cells should be investigated.

CD9 Expression in Non-Hematopoietic Tissues

Murine CD9 expression has also been found in non-hematopoietic tissues. Northern blot analysis has indicated that CD9 is transcribed in the heart, kidney, lung, spleen, brain, nerves, thymus and liver in mice [171, 177, 222]. Western blots confirmed protein expression in heart, kidney, lung, intestine, muscle, spleen and brain [177]. Reports of tissue expression in homogenates of heart and liver should be cautiously interpreted since these preparations may be contaminated with blood cells. The significance of CD9 expression in stromal tissues is uncertain. Treatment of stromal or myeloid cells with anti-CD9 antibodies enhances adhesion of these cells and results in inhibition of migration, suggesting that signaling through CD9 augments the adhesive properties of both cell types. Recent publications correlated the expression of CD9 to the ability of certain tumors to metastasize [223]. Interestingly, uterine invasion by the placenta has been equated to metastatic invasion of cancer cells, although placental invasion is generally a controlled process. The possible role of CD9 in invasion of the murine placenta should be investigated. The expression of CD9 by the anchoring placental structures suggests that this tetraspanin could regulate endometrial invasion. CD9 is expressed in invasive trophoblast cells in humans. If CD9 is also expressed on

similar cells in mice, PSG17 induction of IL-10 through interaction with CD9 could suppress uterine invasion by trophoblast cells.

Signaling Mechanisms

The PSG17N signaling cascade

PGE_2 production by macrophages within 2 hours of PSG17 treatment suggested that synthesis of this eicosanoid is a direct result of PSG17 binding to its receptor. In contrast, upregulation of IL-10 and IL-6 mRNA occurred later, suggesting that de novo protein synthesis was necessary to upregulate mRNA production of these cytokines after treatment with PSG17. The necessity of de novo protein synthesis was confirmed by co-treatment with cycloheximide, which prevented increased production of both IL-10 and IL-6 mRNA. Induction of both IL-6 and IL-10 protein secretion required a minimal dose of 10 μg/ml PSG17N, suggesting that the same receptor is utilized to induce both cytokines. As previously stated, PGE_2 has been shown to induce both IL-10 and IL-6 in activated murine macrophages [214]. PGE_2 biosynthesis is directly mediated by the cycloxygenase enzyme, COX-2. Interestingly, cycloxygenase-2 deficient mice are plagued with reproductive failures [224]. We showed that PSG17 induced PGE_2 within 2 hours of treatment at the same minimal concentration that induced IL-10 and IL-6. Whether the induction of PGE_2 by PSG17 is mediated by Cox-2 will be investigated with pharmacological inhibitors of this enzyme, such as indomethacin, NS-398 or SC-58235. Induction of PGE_2 could provide PSGs with a pivotal means of regulating activated macrophages and systemic cytokine production. In addition, PGE_2 can increase macrophage levels of cyclic-AMP, which in turn activates IL-10 and IL-6 expresssion via

transcription factors such as CREB and AP1. Therefore, PGE_2 regulation in response to PSG17 may be a critical part of the signal transduction pathway leading from receptor activation to cytokine secretion.

CD9 Signaling Events

If PSG17 induction of cytokine secretion through CD9 binding is confirmed, we intend to examine the signaling events involved in this pathway. All of the tetraspanin family members contain four putative transmembrane domains, two extracellular loops and two short cytoplasmic tails. The short cytoplasmic tails of tetraspanins suggest that they may signal through other associated proteins. Identifying the CD9 associated proteins requires gentle extraction to prevent disruption of the association of tetraspanin molecules with other tetraspanins and with integrins [156, 163]. Murine CD9 has been shown to associate with other tetraspanins including CD63, CD81, and CD82 [161]. CD9, CD63, CD81 and CD82 have been found in complex with HLA-DR and VLA integrins as confirmed by immunoprecipitation and immunoblotting of transfected fibroblasts and B lymphoma cell lines [161]. CD9 also associates with various β1 integrin molecules including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_{IIb}\beta_3$ [225-228]. A number of tetraspanins, including CD9, specifically associate with a 55 kDa type II phosphatidylinositol-4-kinase in lymphoid and carcinoma cells [229]. CD9 has also been linked to G proteins but only in platelets [230, 231]. The variety of possible signaling cascades that these associations provide suggest mechanims by which tetraspanins can be linked to their proposed functions of cell motility, growth and cell differentiation. There

is currently no information regarding associations of CD9 with other tetraspanins, cell surface or intracellular molecules in macrophages.

With the almost ubiquitous expression of CD9 on murine hematopoietic cells, one question that arises is "How might the CD9-PSG17 interaction affect stromal cells or other hematopoietic derivatives such as megakaryocytes and platelets?" The presence or absence of signaling components and binding partners within these cells could potentially determine the biological effect of the receptor-ligand interaction. There is a consensus in the literature that CD9 and other tetraspanin protein complexes may be cell type and activation state dependent [154], suggesting that signaling cascades through this receptor are cell-specific. Therefore, until the components of these complexes are determined the effects of PSG17 on various CD9-expressing cells can not be predicted.

Working toward a Comprehesive Mouse Model of PSG Function

The *in vitro* biological effects of murine PSGs are being investigated for the purpose of developing a viable animal model for the study of human PSG function. Our results indicate that despite the structural differences between murine and human PSGs, they both have similar biological functions *in vitro*. The complexity and size of the murine and human PSG families have made the identification of their function very difficult. Although all human PSG cDNAs have been cloned and sequenced, much further research needs to be done with the murine PSG family. Reagents that can identify individual PSG family members may be impossible to obtain due to their similarity at the amino acid level. In the next sections, I will describe the tools our laboratory is developing towards the goal of creating a mouse model of PSG function.

Generation of a Murine Anti-PSG Antibody

Anti-murine PSG antibodies will be useful in characterizing binding and function of these proteins. Preliminary attempts to produce PSG18N-GST antibodies were not successful. Unfortunately, the antibodies developed were not PSG18N-specific and bound to a variety of other denatured proteins on Western blots [232]. Another attempt to produce anti-murine PSG antibody is currently being pursued using recombinant PSG17N-Myc-His proteins. Anti-murine PSG antibodies can be used to characterize the molecular weight of secreted PSGs *in vivo*, and to determine the concentration of murine PSGs in serum throughout gestation. As was observed with human PSGs, we anticipate that murine PSG anitbodies will not be specific for individual PSGs.

Knockouts and Transgenes

Notwithstanding the differences in receptor specificity, some murine PSGs have overlapping biological functions. Therefore, we would expect that the genetic deficiency of a single PSG receptor would not be incompatible with successful pregnancy. Indeed, CD9 knockout females can produce a few viable fetuses [188, 189]. Macrophages from CD9-/- mice will also be useful in confirming that PSG18 and PSG19 use different receptors from PSG17. Because these murine PSGs did not bind to CD9 in *in vitro* experiments, we expect them to induce cytokines in macrophages from CD9-/- mice.

We have already developed a PSG17N transgenic mouse, which will allow us to study *in vivo* PSG17 function outside of the context of pregnancy. We created the transgenic construct by extracting the PSG17N-Myc-His cDNA from the pcDNA3.1 vector and inserting it into pEAK10 (Egde Biosystems). Without anti-murine PSG

antibodies, the myc-his tag was necessary to confirm protein expression *in vivo*. Expression of PSG17N-Myc-His in the pEAK10 vector is driven by the elongation factor 1 alpha promoter (ELF1α). ELF1α is a strong promoter for expression *in vitro* and *in vivo*, and therefore, we anticipate that PSG17N will be expressed in most, if not all, tissues. Ten pronuclei were injected and two founders were identified. The transgenic animals were initially screened for the presence of the insert by PCR. To confirm PSG17 DNA was transcribed in these animals, RNA was extracted from several tissues and analyzed by RT-PCR. PSG17N was transcribed in several organ tissues including lung, heart, kidney, intestines and spleen of progeny from one of the two transgenic founders. Several studies are planned after we confirm the expression of PSG17 protein. PSG17N transgenic mice will be crossed with mice that are susceptible to autoimmune diseases, such as experimental acute encephalitis (EAE). EAE is used as a model for multiple sclerosis, and pregnancy has been shown to affect the severity of this and other autoimmune disorders. This system could be used to determine whether the constitutive production of PSG17 leads to remission or at least repression of symptoms including paralysis. The effects of high levels of PSG17 during the course of an immune response to several well-characterized pathogens would also be interesting to examine. Serum levels of both TH1 and TH2 cytokines should be measured in the transgenic animals, and compared to control mice that had or had not been challenged with pathogens.

Identifying additional human and murine PSG receptors

We are currently pursuing the identity of the receptor(s) for human PSGs. Human and murine CD9 display 89% homology at the amino acid level [187], which suggests

that at least one of the human PSGs might also utilize this binding partner. We have tested recombinant human PSG1, PSG6 and PSG11 proteins for binding to 293T cells over-expressing human CD9, and none of these PSGs associated with this tetraspanin. We are systematically testing for interactions between available human tetraspanin clones and these three human PSGs. The majority of amino acid differences between human and murine CD9 tetraspanins are located in the larger of the two extracellular loops [187], and the expression pattern of CD9 also differs slightly between the two species [233]. Therefore, it is possible that human PSGs have evolved to utilize a different receptor mechanism entirely. Regardless of whether human PSGs bind to a member of the tetraspanin superfamily, the similarities between immune regulatory functions of PSGs in mice and humans justify the use of a mouse model of PSG function. The identification of a tetraspanin family member as a receptor for human PSGs would only strengthen this model.

Investigation of additional receptors linking murine PSGs with immunoregulatory function will help us to define the role of individual murine PSGs during pregnancy. To identify other murine PSG binding sites, a graduate student in our lab is currently screening the RAW cell expression library for the murine PSG19 receptor. Treatment of RAW cells with PSG19N-Myc-His also increased secretion of IL-10, IL-6 and $TGF\beta_1$, indicating that activation of a different PSG receptor(s) induces similar biological functions in these cells. We are also continuing to screen for binding of murine PSG18 and PSG19 to murine tetraspanin family members. The tetraspanin family members, CD53 and CD81 are only expressed in specific hematopoietic cells, which makes them interesting potential PSG targets [155].

Murine PSG17 binds to the tetraspanin CD9. This is the first interaction defined for both of these glycoproteins. The tools for determining the PSG signaling cascade as well as the biological function of a tetraspanin family member, are now at our disposal. We will continue to pursue the identification of other PSG receptors. Generation of a PSG transgenic mouse and identification of the interaction between PSG17 and CD9 has provided us with the tools necessary to design *in vivo* studies in the future. This information is vital for dissecting the mechanisms by which PSGs induce cytokine mRNA and exert any other potential effects on the maternal immune system.

V. Appendix 1

PSG Nomenclature

Current literature reviews and internet searches reveal that a variety of names are in use for PSG genes and proteins. Human PSGs are referenced as human pregnancy –specific β1-glycoprotein (PSβG or PBG), (very frequently) Scwangerschaftsprotein 1 (SP1), pregnancy-associated plasma protein C (PAPP-C) and trophoblast-specificβ1-glycoprotein (TSG). Murine PSGs are called murine Carcinoembryonic antigens (*mCea*), and rat PSGs are referred to as *rnCGM*. Several proposals for new PSG nomenclature have been recommended [234, 235] in attempts to standardize the incongruent identification of PSGs in this growing field of research. To avoid confusion in this manuscript, all references reflect the November 2, 1999 CEA nomenclature announcement published in Experimental Cell Research **252**(2) 243-249. PSG family members are referred to as PSGs followed by a gene identification number. Human PSGs genes are designated *PSG1-11*. Mouse PSG genes are now named *PSG16-29* and rat PSG genes follow as *PSG36-40*. Italics are used to indicate the gene and normal font indicates the corresponding protein.

VI. Appendix 2

Purification of PSG17N-Myc His

PSG 17N Myc-His/pFastBac was transfected into *Sf*9 cells with Cellfectin (Life Technologies). The virus was titered by plaque assay according to manufacturer's instructions (Life Technologies). To produce protein, Hi5 cells were inoculated with a multiplicity of infection of 1 in Ex-400 Media (Irvine Scientific), and the cell supernatants were harvested 72 hours post infection. Protease inhibitors (1% aprotinin, 1% leupeptin, 1% phenylmethylsulfonyl fluoride (PMSF) Sigma) were added to prevent protein degradation. Insect cell supernatant containing recombinant secreted protein was concentrated with an Amicon Ultrafiltration 8400 Cell and YM-3 ultrafilter (Millipore). The concentrated supernatant was dialyzed in 300 mM NaCl; 50 mM NaH_2PO_4 dialysis buffer using snakeskin dialysis tubing (MWCO 10,000 Daltons)(Pierce). PSG17N-Myc His was precipitated from the dialyzed supernatant with Ni-NTA beads (Qiagen) in the presence of 10mM imidazole in dialysis buffer to decrease non-specific binding. Bound proteins were eluted from the beads with increasing concentrations of imidazole. The eluted proteins were concentrated in a Centriprep-3 (Millipore).

The recombinant protein was then separated by electrophoresis in a 12% SDS PAGE gel in a model 491 Prep cell apparatus (BioRad) and collected in test tube fractions as they migrated out of the gel. PSG17N-Myc-His-containing fractions, as determined by Western blot with anti-myc Ab, were pooled and electroeluted in 10 mM Tris buffer, pH 8.0 in a Sialomed apparatus (Amika Corp., Columbia, MD) to remove SDS from the sample. The proteins were concentrated with a centriprep-3 (Millipore). The final protein concentration was ascertained by comparison to bovine serum albumin

standards on a 4-12% Bis Tris SDS-PAGE gel (Invitrogen) stained with GelCode blue reagent (Pierce-Endogen) using the Eagle Eye II Still Video system and Eagle Sight version 3.1 software (Stratagene). Limulus amebocyte lysate assays (Biowhittaker, MA) were performed to ensure low levels of LPS contamination. Protein samples were deemed negative only used if endotoxin levels were less than 0.2 ng/ml which would not result in increased cytokine secretion in macrophages (Dr. Stephanie Vogel, USUHS, *personal communication*).

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